THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Zbtb46 and Runx3 regulated blood cell differentiation from pluripotent embryonic stem cells

by Pál Botó

Supervisor: István Szatmári PhD

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR CELL AND IMMUNE BIOLOGY

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Contents

1. Lists of abbreviations

2-ME – β-mercaptoethanol

α-MEM – alpha modified minimum essential medium

BATF3 – basic leucine zipper ATF-like transcription factor 3

BCL6 – B cell lymphoma 6; also known as ZBTB27

BCL11A – B cell lymphoma 11A

BCOR – BCL6 corepressor

BDCA1 – blood dendritic cell antigen 1

BDCA3 – blood dendritic cell antigen 3

BM – bone marrow

BrdU – 5-bromo-2'-deoxyuridine

BTB – broad complex, tram track, bric-a-brac

CAR – chimeric antigen receptor

CAS9 – CRISPR-associated protein 9

CBF-β – core binding factor-β

CCL19 – C-C motif chemokine ligand 19

CCL21 – C-C motif chemokine ligand 21

Ccr7 – C-C motif chemokine receptor 7

cDC – conventional dendritic cell

CDX4 – caudal type homeobox 4

CRISPR – clustered regularly interspaced short palindromic repeats

CTLA-4 – cytotoxic T-lymphocyte-associated protein 4

DC – dendritic cell

DMEM – Dulbecco's Modified Eagle Medium

DMSO – dimethyl sulfoxide

DPBS – Dulbecco’s phosphate-buffered saline

DTR – diphtheria toxin receptor

E2-2 – SL3-3 enhancer factor 2; also known as TCF4

EB – embryoid body

EBD – EB differentiation

Esam – endothelial cell-selective adhesion molecule

ESC – embryonic stem cell

FBS – fetal bovine serum

Flk1 – fetal liver kinase 1

Flt3 – FMS (Feline McDonough Sarcoma)-like tyrosine kinase 3

Flt3L – FMS-like tyrosine kinase 3 ligand

G418 – geneticin

GATA1 – GATA-binding factor 1

GEMM – granulocyte, erythrocyte, macrophage and megakaryocyte

GM – granulocyte and macrophage

GM-CSF – granulocyte-macrophage colony-stimulating factor

HDAC – histone deacetylase

HLH – helix-loop-helix

HOXB4 – homeobox B4

hPSC – human pluripotent stem cell

HSC – Hematopoietic stem cell

Ikzf1 – Ikaros family zinc finger protein 1

Id2 – inhibitor of DNA binding 2

IL-3 – interleukin-3

IL-6 –interleukin-6

IL-12 – interleukin-12

IFN-α – interferon alpha

IFN-γ – interferon gamma

IMDM – Iscove's Modified Dulbecco's Medium

iPSC – induced pluripotent stem cell

IRF4 – interferon regulatory factor 4

IRF8 – interferon regulatory factor 8

KLF1 – Kruppel like factor 1

LC – Langerhans cell

LDB1 – LIM domain binding 1

LIF – leukemia inhibitory factor

LMO2 – LIM Domain Only 2

LPS – lipopolysaccharide

MACS – magnetic cell separation

MHC – major histocompatibility complex

MLR – mixed leukocyte reaction

MDP – monocyte and dendritic cell precursor

MEF – mouse embryonic fibroblast

Mx1 – MX dynamin like GTPase 1

NCOR – Nuclear Receptor Corepressor 1

NeoR – geneticin resistant gene

NFIL3 – Nuclear factor, interleukin 3 regulated

NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells

NK cells – natural killer cells

NOD – Nucleotide-binding and oligomerization domain

OVA – ovalbumin

PAMP – pathogen-associated molecular patterns

PD1 – Programmed cell death protein 1

PD-L1 – Programmed death-ligand 1

pDC – plasmacytoid dendritic cell

PDGFRα – platelet-derived growth factor receptor alpha

PGK – Phosphoglycerate Kinase

PRR – pattern recognition receptor

PSC – pluripotent stem cell

PU.1 – purine rich box-1; also known as Spi-1

RBPJ – recombination signal binding protein for immunoglobulin kappa J region

RELB – v-rel reticuloendotheliosis viral oncogene homolog B

RPMI – Roswell Park Memorial Institute Medium

RT-PCR – reverse transcription PCR

rtTA – reverse tetracycline transactivator

RUNX1 – runt-related transcription factor 1

RUNX2 – runt-related transcription factor 2

RUNX3 – runt-related transcription factor 3

sgTRE – second generation tetracycline response element

Spi-B – Spi-1/PU.1 related transcription factor

STAT – Signal transducer and activator of transcription

TGF-β – transforming growth factor beta

Th1 – T helper type 1

Th17 – T helper type 17

TNFα – tumor necrosis factor alpha

TrkC – tropomyosin receptor kinase C

ZBTB7B – zinc finger and BTB domain containing transcription factor 7b; also known as ThPOK
ZBTB46 – zinc finger and BTB domain containing transcription factor 46; also known as BTBD4 or zDC
ZF – zinc finger

2. Keywords

Embryonic stem cell, pluripotent cell, dendritic cell, transcription factor, differentiation, reprogramming, cell fate determination

2. Introduction and literature review

2.1 Introduction

Immunotherapy represents a powerful weapon to treat solid tumors and hematopoietic malignancies. Moreover, this approach can be applied to mitigate autoimmune diseases and inflammatory disorders. For example, immune checkpoint inhibitor antibodies can be used to promote the T cell response by antagonizing co-inhibitory signals (e.g., PD1/PD-L1 or CTLA-4). Interestingly, durable immune responses were detected in numerous types of tumors upon the application of these checkpoint blocker antibodies (Robert et al., 2011; Zou et al., 2016). Moreover, adoptive cell therapies can be applied using tumor-infiltrating lymphocytes or peripheral T cells. These lymphocytes can be engineered to target cancer-specific antigens with T cell receptors or chimeric antigen receptors (CARs) (Chodon et al., 2015). It is worth mentioning that clinical trials with CD19-targeting CAR T cells have shown a 90% remission in patients with acute B lymphoblastic leukemia, indicating the great potential of this cell-based therapeutic approach (Lim and June, 2017). More related to this study, that *ex vivo*-produced dendritic cells (DCs) can be loaded with tumor antigens and injected back into cancer patients to stimulate the antitumor immune response. Some of these DC-based vaccines have shown promising clinical results (Vacchelli et al., 2013).

DCs are usually generated by *in vitro* differentiation of peripheral blood monocytes for immunotherapy. However, there is a limitation in the number of the harvested monocytes, and the DC-differentiation capacity of these cells varies depending on the blood donor (Senju et al., 2011). In contrast to the monocytes, the pluripotent embryonic stem cells (ESCs) can provide an inexhaustible source for immune cell therapies because of their unlimited self-renewal activity and broad differentiation capacity. ESC-derived DCs can be made through *ex vivo* differentiation using well-defined protocols (Murry and Keller, 2008; Senju et al., 2011). However, it is still challenging to steer the differentiation of ESCs to functional cells because the end products often represent embryonic type or immature cells. In ESC-derived cells the embryonic developmental programs are readily activated, however, these gene regulatory networks usually do not guarantee the formation of fully mature cells (Chang et al., 2006; Hrvatin et al., 2014). For proper maturation, further steps are needed which are missing from the existing *in vitro* differentiation protocols.

Genetically encoded tools like ectopically-expressed transcription factors allow cell engineers to enhance the maturation of the *ex vivo* generated immune cells. For example, the development of the pluripotent stem-cell–derived blood cells were modified with ectopically expressed

transcription factors (Doulatov et al., 2013; Elcheva et al., 2014; Kyba et al., 2002; Sugimura et al., 2017; Wang et al., 2005; Yamamizu et al., 2013). In my research project, I carried out a similar approach, the effects of two DC specific transcription factors (RUNX3 and ZBTB46) were investigated during the *ex vivo* differentiation of the pluripotent ESCs.

2.2 Literature review

2.2.1 Dendritic cells

DCs represent a relatively rare population of immune cells with bone marrow origin; these special antigen presenting cells modulate broad range of immune functions in human and mouse (Mildner and Jung, 2014). DCs are specialized to capture, process and present antigens. These immune cells can reside at the mucosal and internal surfaces as well as at the skin; maturation process triggers their migration to lymphoid organs to initiate antigen specific immune response. DCs recognize pathogen-associated molecular patterns (PAMP) using pattern recognition receptors (PRR). Different types of PRRs can contribute to this mechanism e.g., Toll-like receptors, NOD-like receptors and C-type lectin receptors (Van Brussel et al., 2012). DCs can utilize several types of cellular mechanisms to capture the recognized antigens including phagocytosis, pinocytosis and endocytosis (Palucka and Banchereau, 2012; Steinman and Banchereau, 2007). Protein antigens are processed into peptides inside the DCs, then presented on major histocompatibility complex (MHC) I and II molecules. Moreover, the non-classical MHCI molecules play an important role in the presentation of non-protein e.g., lipid antigens (Palucka and Banchereau, 2012). The antigen presentation of DCs can be divided into three subcategories. DCs present the processed exogenous antigens to CD4+ T cells with the MHCII complexes. Endogenous antigens are presented to CD8+ T cells utilizing MHCI complexes on their surface. Finally, some DC subtypes have the capacity to the cross-presentation of exogenous antigens in their surface using MHCI complexes to CD8+ T cells (Constantino et al., 2017).

DCs have historically been defined as a distinct cell lineage based on their properties such as their cell surface markers (CD11c and MHCII expression), their morphology (the presence of dendrites) and their immune function (the ability to present antigens and to stimulate naïve T cells) (Perie and Naik, 2015). Moreover, DCs can be identified by the lack of specific markers of other blood cell lineages such as B cell (CD19, CD20), monocytes (CD14), stem cells (CD34), T cells (CD3) and NK cells (CD56). As a matter of fact the terminology of ‘DCs’ describes a rather heterogeneous population of immune cells including plasmacytoid (pDC), conventional (cDC), Langerhans cells (LCs) as well as monocyte-derived DCs (Satpathy et al.,

2012b). The unique feature of pDCs to produce type I interferons upon viral infection makes this cell type a key component of the innate immune responses. Well-known function of the cDCs is to capture and process antigens from bacterial origin. Upon antigen stimulation this cell type produces pro-inflammatory cytokines (e.g., IL6, IL12 and TNFα), thus gathering cytotoxic T cells and activating Th1 and Th17 pro-inflammatory T cell subsets (Balan et al., 2019; Constantino et al., 2017; Steinman and Banchereau, 2007). The cDCs can be further divided into two major subtypes: cDC1s (CD8+ and CD103+ DCs in mice, BDCA3 +DCs in humans) and cDC2s (CD11b+ CD4+ CD8− DCs in mice, and BDCA1+ CD1c+ DCs in humans) (Perie and Naik, 2015). In addition, in mice, monocytes can be converted to DC like cells *in vivo* under inflammatory conditions, or after *ex vivo* treatment with GM-CSF (granulocyte-macrophage colony-stimulating factor) (Briseno et al., 2016). Similarly, human monocytes cultured *ex vivo* with GM-CSF and IL-4 also acquire DC characteristics (Sallusto and Lanzavecchia, 1994). Finally, LCs can be also classified as a DC subtype because these immune cells are capable of antigen presentation. The other unique function of LCs compared to the classical macrophages the migratory potential to lymph nodes, but their gene expression signatures are more related to the macrophages (Miller et al., 2012).

2.2.2 DC development

Hematopoietic stem cells (HSCs) can differentiate into myeloid and lymphoid progenitors. *In vivo*, most of the DC subsets are derived from common myeloid progenitors that can develop into monocyte and dendritic cell precursors (MDPs) (Fogg et al., 2006). The next distinct stage of the DC developmental program is the generation of the common dendritic cell progenitors (CDPs) which are the precursors of the pDC and cDC subsets (See et al., 2017). It seems that the *in vivo* DC development is more complicated because single cell fate mapping studies have uncovered strong variability in early BM progenitors that give rise to DCs (Naik et al., 2013). A number of cytokine signals modulate the DC differentiation through the progenitor stages. The major cytokine receptor regulating DC development is Flt3 (fms like tyrosine kinase 3), which can be detected on many early blood cells precursors and remain highly expressed on DC progenitors. Flt3 and its ligand (Flt3L) play a major role in the cDC and pDC differentiation process, in the absence of this receptor or its ligand an impaired DC generation can be observed (McKenna et al., 2000). The GM-CSF receptor and its ligand also play a prominent role during DC differentiation, a strong impairment of DC generation was observed in GM-CSF receptor deleted mice (Vremec et al., 1997). Furthermore, GM-CSF drives inflammatory DC development from monocytes in mice (Briseno et al., 2016).

2.2.3 DC generation from adult progenitors and applications for immunotherapies

In vivo DCs are a sparsely distributed, rare immune cell types, however, functional DCs can be differentiated *ex vivo* from more frequent progenitors. There are several approaches to generate functional DCs for immunotherapies. Many cases mononuclear cells of the peripheral blood are used to DC generation obtained from a patient (autologous cells). One of these is the selection of CD14+ monocytes and their cultivation in the presence of IL-4 and GM-CSF (Sallusto and Lanzavecchia, 1994). This approach has been proven to be effective in multiple diseases (Florcken et al., 2013), but also an expensive and time-consuming process. CD11c+ cells from umbilical cord-blood or allogenic DCs donated by healthy volunteers can also be utilized (Kumar et al., 2015; Pinzon-Charry et al., 2005). Another alternative source of DC is the mobilized CD34+ stem and progenitor cells from the BM then treated with a cocktail of cytokines e.g., GM-CSF, Flt3L and TNFα (Redman et al., 2008). It is also possible to expand the circulating DCs *in vivo* using Flt3L treatment; these antigen presenting cells are an efficient activator of the T cell responses (Sabado et al., 2017). Among these approaches DC generation from monocytes purified from whole blood is by far the most commonly applied method.

Maturation is a crucial step of the functional DC generation process to obtain the capacity to activate immune responses for therapeutic purposes. The maturation of the monocyte derived DCs then can be induced by specific set of cytokines thereafter the mature DCs can be injected into the patient (Sabado et al., 2017). Lipopolysaccharide (LPS), CD40 ligand (CD40L), IFN-γ, tumor necrosis factor-α (TNF-α) and IFN-α can be applied to induce DC maturation (Castiello et al., 2011). Maturation of the DCs *in vivo* using antigen coupled antibodies could be a cost-efficient alternative strategy to the current ex vivo DC generation process. To find the proper combination of the required stimuli however is still in the scope of numerous researchers (Nicolette et al., 2007; Sabado and Bhardwaj, 2010).

Autologous DCs generated *ex vivo* could be primed with tumor antigens then delivered into patients with malignant disease for anticancer therapy. DCs, created *ex vivo* form monocytes or CD34+ progenitors have been shown to promote antigen-specific T cell responses in both preclinical and clinical studies. The very first cell-based therapy to hormone-refractory prostate cancer approved by the FDA (US Food and Drug Administration) was the Provenge (Sipuleucel-T), a CD54 enriched vaccine derived from peripheral blood. This vaccine showed promising potential in Phase I-III clinical trials (Kantoff et al., 2010; Sabado et al., 2017). In summary, DC therapies have been the subject of several clinical studies testing various cell therapeutic strategies against tumors. It should be mentioned, that despite the initial enthusiasm,

disappointing results from many trials raised doubts regarding the clinical value of these methods. However, the expanding knowledge on DC differentiation and maturation have allowed a more successful approach of DC-based immunotherapies (Constantino et al., 2016). There are many protocols for DC-based vaccination (Figure 1).

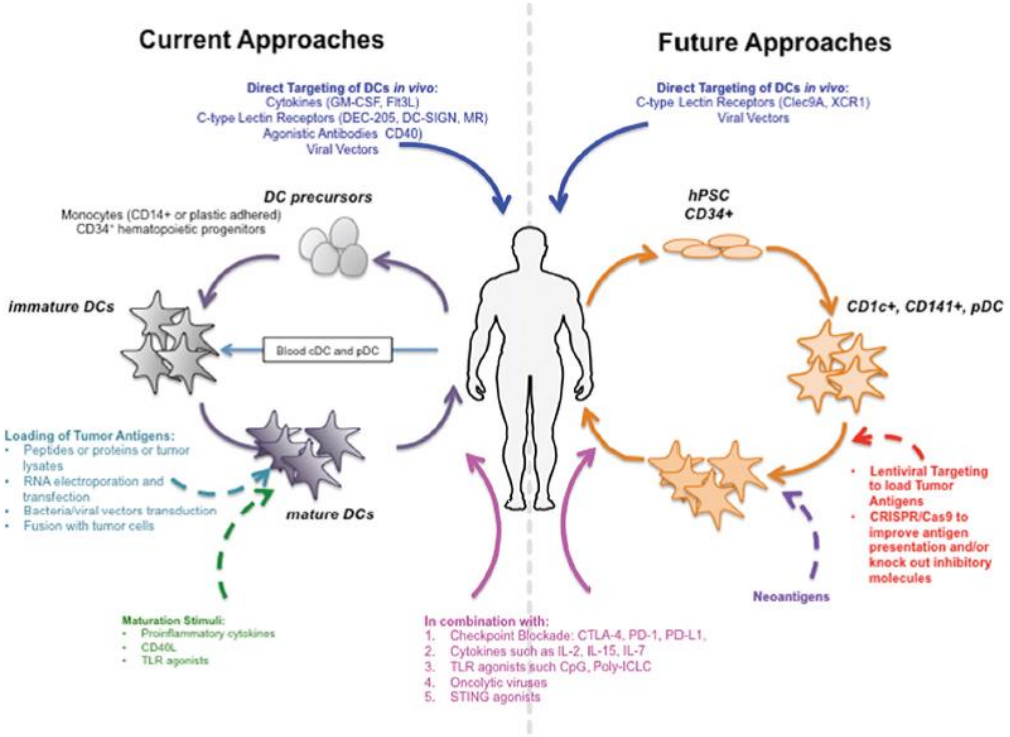

Figure 1. Current and future approaches for DC immunotherapies (Sabado et al., 2017). A possible future approach is the production of genetically modified DCs from human pluripotent stem cells (hPSCs).

It is also worth mentioning that, only a limited number of monocyte-derived DCs can be generated, therefore it would be better to produce DCs from the limitless source of pluripotent stem cells (PSCs). ESCs or iPSCs (induced pluripotent stem cells) have the potential to give rise to any cell types, raising exciting prospects to generate DCs from this cell source. In the following sections I will introduce the main characteristics of the PSCs and their *ex vivo* differentiation into DCs.

2.2.4 Pluripotent stem cells for blood cell differentiation

PSCs can be found naturally in the mammalian blastocyst and constitute the founder cells of the embryo proper (Theunissen and Jaenisch, 2017). There are two major types of *ex vivo* cultured PSCs: ESCs and iPSCs. ESC lines can be established from the inner cell mass of the early-stage blastocyst (Evans and Kaufman, 1981; Martin, 1981). On the other hand, iPSCs can be dedifferentiated from somatic cells by reprogramming (Takahashi and Yamanaka, 2006). Two major attributes of the PSCs are the self-renewal (unlimited capacity of proliferation) and pluripotency, the ability to differentiate into various cell types of the three germ layers,

ectoderm, endoderm and mesoderm (Wobus and Boheler, 2005). There are several human and murine PSC model systems. Human PSCs have become an important platform to develop cell therapeutics for regenerative medicine, to screen compounds for drug discovery and to generate disease models (Cherry and Daley, 2012). In parallel, murine PCS are still useful research tools because murine ESCs represent the ground-state (naïve) pluripotency (De Los Angeles et al., 2015). Moreover, the mouse ESCs/iPSCs can be easily disaggregated and readily form new colonies, therefore they are more suitable for genetic manipulation comparing with the human counterparts. In this thesis report I used mouse ESC derived blood cells and their progenitors, therefore here I focus on the murine PSC culture and differentiation.

Mouse ESCs can be maintained indefinitely on mitotic arrested mouse embryonic fibroblast (MEF) feeder cell layers in the presence of leukemia inhibitory factor (LIF) and fetal bovine serum (FBS) (Smith et al., 1988; Williams et al., 1988). Moreover, murine ESCs can be maintained in serum-free medium with two small-molecule inhibitors (2i) (Ying et al., 2008). Removing these supportive agents (LIF and/or 2i), ESCs can be differentiated *in vitro* into any cell types of the 3 germ layers using adequate culture conditions.

There are multiple approaches to control the early ESC differentiation toward mesodermal and blood cells: embryoid body (EB) formation, feeder-cell based co-culture method, or ESCs can be grown in extracellular matrix coated dishes (Pratumkaew et al., 2021). The EBs form 3D aggregates of ESC derived cells, support the three germ layers development but under conditions of 15% FBS containing medium, the major germ layer represented is mesoderm (Keller et al., 1993; Kyba et al., 2002). The hanging drop method is an efficient way of EB formation and widely used to establish ESC derived mesodermal cells and hematopoietic progenitors (Kyba et al., 2002; Szatmari et al., 2010). Another well-known method is using feeder cells in co-culture with ESCs to support the mesoderm and blood cell development. OP9 stromal cells are known supporters of the mesodermal and myeloid differentiation of the murine ESCs (Suzuki and Nakano, 2001). Finally, tissue culture dishes coated with vitronectin, fibronectin or collagen can also be used to support the hematopoietic lineage commitment of the ES derived cells (Pratumkaew et al., 2021). During the blood cell development, the mouse ESCs differentiate into Flk1+PDGFRα− cells, which are precursor of endothelial and hematopoietic cells. These precursors cell can be converted to HSC-like cells or to functional erythroid, myeloid or lymphoid cells (Inoue-Yokoo et al., 2013). In the next section I will discuss the ESC derived DC differentiation.

2.2.5 DC generation from pluripotent stem cells

First, Fairchild and his coworkers described a protocol for generation of functional DCs from mouse ESCs (Fairchild et al., 2000). In that paper, EBs were first formed, and subsequently differentiation of DC was governed by addition of GM-CSF and IL-3 cytokines. A few years later Senju and his colleagues established an other method to manufacture DCs from mouse ESCs using OP9 feeder cells (Senju et al., 2003). In this protocol ESCs were transferred onto OP9 cell layers and differentiated for 5 days to mesoderm cells in medium containing high concentration of FBS without exogenous cytokines. To induce differentiation to myeloid blood cells, the 5-day differentiated cells were harvested and further cultured on fresh OP9 cell layers in the presence of GM-CSF. On day 11 the floating or loosely adherent myeloid cells were harvested and transferred to new dishes without OP9 feeder layers and further cultivated in the presence of GM-CSF. DC like cells were harvested at day 19 (before collecting these cells could be activated with LPS or TNFα). Importantly these DC-like cells had a capacity to process and present antigens and these cells were able to stimulate allogeneic T cell responses (Senju et al., 2003). A follow-up study revealed that these ESC-derived DCs can be genetically modified for example with ovalbumin (OVA) plus chemokine expression vectors to initiate a strong OVA-specific T cell response (Matsuyoshi et al., 2004). In addition, there research group also generated DC like cells from mouse iPSCs (Senju et al., 2009). In parallel with the animal studies, human ESC-derived DCs were also generated and characterized (Senju et al., 2007; Slukvin et al., 2006; Tseng et al., 2009; Zhan et al., 2004). Although, the majority of these papers claimed that ESC-derived DCs are functionally equivalent with the monocyte- or BM-derived DCs. It was also mentioned that, DCs generated form ESCs or iPSCs exhibited lower T-cell activation potential than the adult counterparts, indicating that the impaired immunity can be a general characteristic of the pluripotent stem cell derived DCs (Senju et al., 2003; Tseng et al., 2009; Zhang et al., 2015). To improve the DC specific immune response, we can apply novel combination of cytokines or transcription factor mediated cellular programing. In the following sections I will described the transcriptional regulation of the DC development and the direct application of transcription factors for DC generation.

2.2.6 Transcriptional regulation of the DC development

In cooperation with the cytokine signaling, numerous transcription factors have been detected and characterized upon the formation of the DC lineages (Geissmann et al., 2010; Murphy et al., 2016; Satpathy et al., 2011; Watowich and Liu, 2010). The investigation of knock out mice has revealed the functional importance of several of these transcription factors in DC

development. Some of them affecting multiple DC subsets and exert their effects also on DC progenitors. For example, the transcription factor Ikaros (Ikzf1) is involved in the regulation of several hematopoietic lineages, Ikzf1 deficient BM was shown to fail generating cDCs (Merad et al., 2013). ETS (E-twenty-six) family member PU.1 (also known as Spi-1) is a master regulator of the hematopoietic development, deeply involved in the DC specification. Absence of PU.1 results in general reduction of most immune cell types including macrophages and DCs (Carotta et al., 2010). The signal transducer and activator of transcription (STAT) protein family is a key mediator of cellular functions in DCs. STAT3 is a core regulator of DC commitment through the Flt3 pathway. STAT3 deletion results in reduced pDC and cDC formation (Merad et al., 2013).

Numerous transcription factors more or less selectively regulate the formation of the various DC subsets. The cDC1 subset requires the following transcription factors for development: BATF3, IRF8, NFIL3, Id2 and BCL6 (Murphy et al., 2016). For example, the basic leucine zipper transcription factor ATF-like 3 (Batf3) is generally expressed in all DC subsets, however impaired cDC1 generation was observed as a result of the deletion of Batf3 gene (Hildner et al., 2008). IRF8 is a multifunctional/complex regulator of myeloid differentiation. Although, deletion of the *Irf8* results in/causes severe myeloproliferative disorders; yet this protein is required for the cDC1 development suggesting a key function at both early and late steps of myeloid differentiation (Merad et al., 2013). Inhibitor of DNA Binding Protein 2 (Id2) transcription factors are members of the HLH family inhibit the DNA binding of other HLH family members. Id2 deficiency is associated with the strong downregulation of cDC1 subset (Kusunoki et al., 2003). The cDC2 cells are characterized by expression of the IRF4 rather than IRF8 and subtypes of cDC2 are dependent either on the transcription factors Notch2 (Notch homolog protein 2) or KLF4 (Kruppel-like factor 4). In addition, the development of this DC subset is regulated by RBPJ (Murphy et al., 2016). pDC development is regulated by the following transcription factors: IRF8, E2-2, BCL11a, RUNX1 and RELB (Murphy et al., 2016). For example, RELB, a member of the NF-κB pathway transcription factors influences the pDC development. (Watowich and Liu, 2010). Moreover, Id2 and E2-2 are both belong to the HLH family, E2-2 can control gene expression in pDCs and these factors also can mutually antagonize each other (Merad et al., 2013).

2.2.7 Transcription factor mediated blood cell and DC differentiation

Overexpression of transcription factors has become a feasible method to modify the identity of the cells, especially after the discovery that some of these transcription factors could reprogram

differentiated cells into iPSCs (Takahashi and Yamanaka, 2006; Yamanaka, 2012). This finding has altered fundamental ideas on the stability of cell identity, stimulating novel cell reprogramming approaches including direct-lineage conversions (Chambers and Studer, 2011; Xu et al., 2015). For example, fibroblasts can be transdifferentiated to cardiomyocytes or neurons with a mixture of cardiac- or neuronal-specific transcription factors, respectively (Ang and Wernig, 2014; Srivastava and DeWitt, 2016). Interestingly, fibroblasts can be converted to blood cell progenitors with transcription factor-dependent transdifferentiation (Batta et al., 2014; Feng et al., 2008; Pereira et al., 2013; Szabo et al., 2010). More related to this study that mouse and human fibroblasts were also converted to DC like cells via ectopic expression of PU.1, IRF8 and BATF3 transcription factors. These transdifferentiated immune cells acquired a cDC1-like gene expression profile with the ability to process and present antigens to T cells (Rosa et al., 2018). It was also described that activation of the transcription factor PU.1 in transformed chicken myeloid progenitors promoted DC formation, in addition, PU.1 also triggered the DC fate in human myeloid progenitor and monocyte clones (Bakri et al., 2005).
Importantly, ESC differentiation into hematopoietic cells can also be driven by forced expression of transcription factors. For example, ectopic expression of HOXB4 or CDX4 enhanced the blood cell formation and facilitated the hematopoietic engraftment *in vivo* (Kyba et al., 2002; Wang et al., 2005). Moreover, overexpression of PU.1 shifted the transcriptome of mouse ESCs toward blood cells (Yamamizu et al., 2013). Of note, there are several additional transcription factors which can modify the hematopoiesis and the DC differentiation. In this study I investigate the effects of RUNX3 and ZBTB46 during the ESC-derived DC development and hematopoiesis. In the following sections, therefore, I will describe the role of the RUNX3 and the ZBTB46 transcription factors on the DC and blood cell development.

2.2.8 RUNX transcription factors

Runt-related transcription factors (RUNXs) are expressed in all metazoan animals. In mammals three RUNX genes were identified, RUNX1, RUNX2 and RUNX3 respectively (Hughes and Woollard, 2017). The RUNX proteins contains a ‘runt domain’, which mediates DNA binding to a specific consensus sequence (TGt/cGGt/c) or its extended version of this sequence (Bowers et al., 2010; Tahirov et al., 2001). RUNX DNA binding sites are often located within gene promoters and enhancers and are implicated in transcriptional repression or activation. Furthermore, this transcription factors interact with the core binding factor-β (CBF-β), a non-DNA-binding RUNX partner, which enhances the affinity and specificity of their DNA binding (Golling et al., 1996; Kagoshima et al., 2007). Despite the overlapping DNA binding sites and

gene expression pattern, RUNX proteins have their own well-defined functions during the mammalian cell and tissue differentiation. For example, RUNX1 is required for definitive blood cell development and one of the most frequent targets of chromosomal translocations in leukemias (de Bruijn and Dzierzak, 2017; Sood et al., 2017). RUNX2 is a master regulator of bone differentiation, and its partial defect is able to cause abnormal bone formation (Komori, 2006; Otto et al., 1997). *Runx3* deficient mice show ataxia due to the loss of TrkC proprioceptive neurons in the dorsal root ganglia (Inoue et al., 2002; Levanon et al., 2002). Moreover, RUNX3 is required for the proper development of many immune cell lineages and this protein also participates in the regulation of the early hematopoiesis. In the next section I will introduce the role of the Runx3 during early hematopoiesis and DC formation.

2.2.9 RUNX3 dependent early hematopoiesis and DC development

In contrast to Runx1, Runx3 exhibits a low expression in HSC precursors, however, it has a similar expression pattern to Runx1 in adult and fetal liver HSCs (Landry et al., 2008). de Bruijn showed that Runx1 gene depletion results in embryonic lethal phenotype, however, this was not observed in Runx3 null embryos (de Bruijn and Dzierzak, 2017). Wang showed the lethal consequences in Runx1/Runx3 double KO mouse due to myeloproliferative disorder and the mechanism behind this phenomenon was identified: the loss of the two Runx factors resulted in impaired DNA repair mechanism and genomic toxicity (Wang et al., 2014). Moreover, elevated proliferative potential was observed in *Runx3* (Mx1-Cre) single KO mouse and manifestation of myeloproliferative disorders was detected in older animals (Wang et al., 2013). In Zebrafish an impaired hematopoiesis was described in runx3-depleted embryos, contrary, forced expression of runx3 resulted in the increase of Runx1 expressing progenitor cells (Kalev-Zylinska et al., 2003). In conclusion, these data suggest the definitive role of RUNX1 in blood cell development and highlights that RUNX3 can act together with RUNX1 and further modulate the early hematopoietic cell specification.

Loss of function studies indicate the critical role of RUNX3 for the differentiation of cytotoxic T cells (Wong et al., 2011). In addition, T helper cells, DCs, natural killer cells and B cells are also regulated by RUNX3. We have recently prepared a review article about the RUNX3 regulated immune cells (Boto et al., 2018), here, I only analyze the putative role of this transcription factor on DC development and activation. *Runx3* has a well-defined function in the activation of cDC2: *Runx3*-depletion in DCs of the spleen shows a lowered MHCII expressing and T cell activating phenotype, especially the impairment of Esam++ cDC2 subtype was observed (Dicken et al., 2013). Furthermore, forced expression of RUNX3 resulted

enhanced CD11a/CD18 and CD49d expressing cells and superior functions in T cell priming, migratory potential and antigen presentation (Dominguez-Soto et al., 2005). These results indicate the supportive function of RUNX3 on DC maturation. On the other hand, it was also described that in human, forced expression of TGF-β in *RUNX3* ablated bone marrow derived DCs enhanced the maturation and T cell stimulatory functions suggesting RUNX3 can be in interference with the TGF-β driven DC development (Fainaru et al., 2004). Of note, TGF-β is the key regulator of the LC development (Borkowski et al., 1996), therefore LCs could be selectively modulated by RUNX3. Consistent with this notion, one study suggested that *Runx3* null mice entirely lack of LCs (Fainaru et al., 2004). Supporting this observation overexpression of *Runx3* restored the BM derived LC development even without PU.1 (Chopin et al., 2013). Altogether these findings emphasize the important regulatory role of RUNX3 in LC and cDC2 commitment and differentiation (Figure 2).

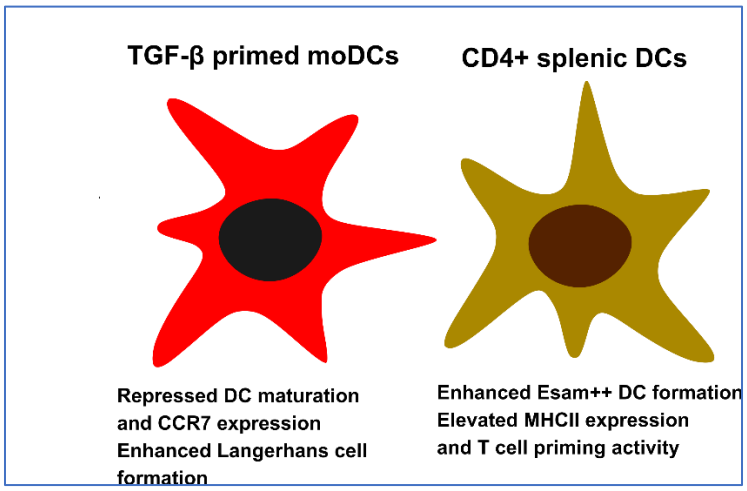

Figure 2. Effects of RUNX3 on DC phenotypes (Boto et al., 2018). The major effects of RUNX3 on TGF-β activated monocyte-derived DCs and in CD4+ splenic DCs (cDC2).

2.2.10 ZBTB protein family

In this study I also investigated the effects of the ZBTB46 transcription factor (also known as BTBD4 or zDC) during hematopoiesis. The ZBTB46 transcription factor is a member of the ZBTB protein family. The ZBTB family consists of almost 60 transcription factors involved in the regulation of cellular functions such as responses to DNA damage, involved in the cell cycle and developmental processes (Zhu et al., 2018). Unique attribute of this family is the highly conserved structural composition, these proteins consist of one or more C-terminal zinc finger domains (ZF) and an N-terminal BTB (broad complex, tram track, bric-a-brac) domain. The ZF domain is responsible for the selective interaction with the target genes via sequence specific binding (Maeda, 2016). The BTB domain is in direct interaction with various co-repressors

such as NCOR-1/2, BCOR, and histone modifying enzymes e.g., HDACs (histone deacetylases), thus has an important role in chromatin-remodeling and gene regulation (Huynh et al., 2000; Zhu et al., 2018). The BTB domain also plays key role in the establishment of protein stability, localization and transcriptional activation of the ZBTB factors (Chaharbakhshi and Jemc, 2016). Moreover, posttranslational modifications can be often detected on the less conserved linker region between the ZF and BTB domains (Maeda, 2016; Zhu et al., 2018).
Several members of the ZBTB family regulates the immune cell specification and function. For example, ZBTB27 (BCL6) was identified as an oncogene associated with B cell lymphomas (Kerckaert et al., 1993), moreover, *Bcl6* null mice exhibit an impaired B cell activation response and severe inflammatory phenotype (Maeda, 2016). ZBTB7B (ThPOK) is associated with the T cell function and lineage commitment: ThPOK deficient mice exhibit a selective absence of CD4+ T helper cells with a concomitant increase in CD8+ cytotoxic T cells (Maeda, 2016). Finally, ZBTB46 is a recently characterized DC affiliated transcription factor; in the following section I will highlight the main characteristics of this ZBTB protein.

2.2.11 ZBTB46 is a DC specific transcription factor

ZBTB46 is a hallmark of the cDC compartment, because this transcription factor is highly expressed in cDCs but missing in pDCs, monocytes and macrophages (Meredith et al., 2012a; Miller et al., 2012; Satpathy et al., 2012a). Moreover, Satpathy et al. also showed that a ZBTB46-driven reporter activity was correlated with a DC-like phenotype in the intact tissue environments (Satpathy et al., 2012a). In addition, Meredith et al. inserted diphtheria toxin receptor (DTR) into the *Zbtb46* gene locus to serve as a genetic tool to deplete the *Zbtb46* expressing cells. Mice bearing this construct express DTR selectively in cDCs and diphtheria toxin treatment into *Zbtb46-DTR* BM chimeras results in cDC depletion (Meredith et al., 2012b). However, further studies uncovered that the expression of *Zbtb46* is not fully restricted to cDCs. ZBTB46 was also detected in LCs and murine monocytes were converted to *Zbtb46* expressing DC-like cells especially in the presence of IL-4 (Briseno et al., 2016; Satpathy et al., 2012a; Zigmond et al., 2012). Moreover, ZBTB46 was also found in erythroid progenitors, HSCs and even endothelial cells (Liu et al., 2020; Satpathy et al., 2012a).
Despite the high expression in cDCs, Zbtb46 deficient mice had a similar number of cDCs in spleens or lymph nodes (Satpathy et al., 2012a). However, high number of monocyte specific and maturation related genes showed higher expression in *Zbtb46* KO cDCs suggesting that without this protein these DCs are partially activated (Meredith et al., 2012b). Moreover, overexpression of *Zbtb46* in BM progenitors resulted in enhanced cDC development but

impaired granulocyte generation. ZBTB46 was shown to govern the DC progenitors towards the cDC identity by lowering the influence of the non-DC growth factors in the cDC development (Satpathy et al. 2012). In addition, ZBTB46 negatively modulates the proliferation of the endothelial cells (Wang et al., 2018). These results demonstrate that, several pathways are affected by ZBTB46 including DC activation and cell proliferation, but more data are necessary to define the function of this recently identified gene regulatory protein.

2.2.12 Transgene induction in pluripotent stem cells and their progenitors

In this study to facilitate the blood cell and DC development we probed DC specific transgenes upon the *ex vivo* differentiation of the genetically modified ESCs. To achieve a uniform gene expression, transgenes can be integrated into a specific chromosomal locus and their expression can be driven by a drug-responsive promoter (chemically inducible transgenes). Several methods are available for targeting a gene of interest into a defined genomic region. For example, homologous recombination can be employed with CRISPR-CAS9 based genome editing (Merkert and Martin, 2016). In addition, recombination mediated cassette exchange can be used on a modified genomic DNA region (Ting et al., 2005; Turan et al., 2013). In this study I used a special inducible cassette exchange recombination approach for site specific transgene targeting (Bencsik et al., 2016; Iacovino et al., 2011; Iacovino et al., 2014). A genetically modified mouse ESC line (ZX1) was used that contains a doxycycline-inducible floxed Cre, which substitutes itself with the transgene upon recombination (Bencsik et al., 2016; Dandapat et al., 2014). During this recombination, a special promoter plus ATG sequences are inserted upstream of the neomycin resistance gene which restores resistance to geneticin (G418) in these ESCs (Figure 3).

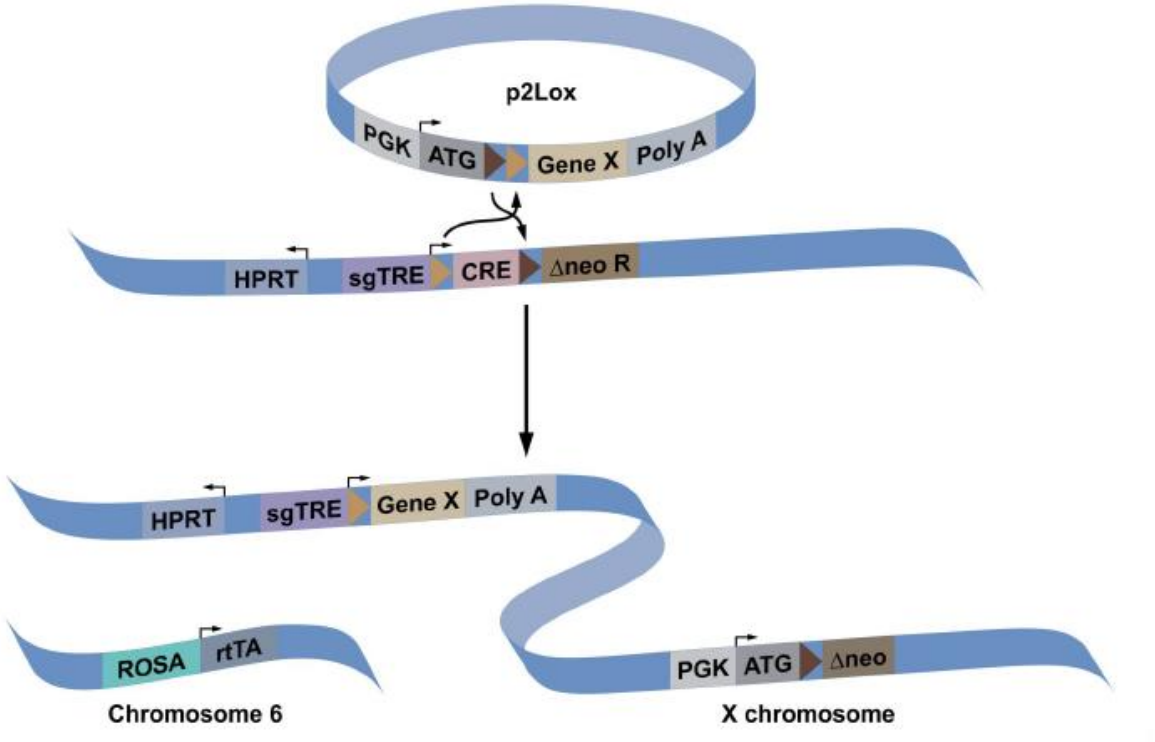

Figure 3. Schematic description of the inducible recombination (Bencsik et al., 2016). The targeting plasmid (p2lox) can be recombined with the target DNA region, which is found upstream of the Hprt gene. In this genomic region a second-generation tetracycline response element (sgTRE) drives the Cre expression. Arrows label recombination between the loxP sites. After recombination the incoming gene is placed downstream of the sgTRE. In parallel, the PGK-promoter plus ATG sequences are introduced upstream of the geneticin resistant gene (NeoR). Of note, the ZX1 ESCs always express a reverse tetracycline transactivator (rtTA).

This inducible system has been previously used to promote the hematopoietic or skeletal muscle development by forced expression of various transcription factors (Darabi et al., 2008; Kyba et al., 2002). Importantly, we have recently found that more uniform transgene induction can be detected with this inducible ESC system by chemical (doxycycline) re-selection or cell sorting (Bencsik et al., 2016).

3. Aims of Study

To improve our understanding on ES-DC differentiation, we carefully examined the efficiency of DC generation from murine ESCs. It was found that ES-DCs showed a lower expression of numerous DC maturation specific markers after LPS treatment compared to BM-DCs. Moreover, some DC specific transcription factors were barely detected in ES-DCs. Based on these data we aimed to examine the effect of two important regulators of the DC development, the RUNX3 and ZBTB46 transcription factors in mouse ESC derived DC progenitors.

We intended to assess the developmental and immunological properties of ES-DCs influenced by these factors in phenotypic level and also perform gene expression analysis to uncover the following:

- Characterize the RUNX3 driven ES-DC maturation process

- Reveal the modulatory role of RUNX3 in T cell activation and migratory potential of the ES-DCs

- Assess how ZBTB46 influences the early ESC-mesoderm transition and the myeloid cell development

- Determine how ZBTB46 influences the cell cycle and proliferation of ESCs

- Reveal how ZBTB46 alters the global gene expression pattern of ESC derived progenitors

- Prove the effect of ZBTB46 in the ESC derived erythroid development.

4. Materials and Methods

4.1 Embryonic stem cell culture, maintenance and cryopreservation

Murine ESCs were cultivated using a previously described (Szatmari et al., 2010) protocol with modifications. In summary, mitomycin C (Merck) treated MEF cells as feeders were utilized for the maintenance of the ESCs in knockout DMEM (Thermo-Fisher Scientific) containing 1000 U/ml LIF (Merck), 15% FBS (Thermo-Fisher Scientific), 100 μg/ml streptomycin and 100 U/ml penicillin (Merck). Cells were harvested with 0.25% trypsin-EDTA (Gibco) solution and cells were frozen using 10 % DMSO (Sigma Aldrich) containing cell culture medium.

For expansion and reselection of transgenic cells before differentiation mouse ESCs were maintained without MEF feeder cells using 0.1% gelatin coated 6 well tissue culture plate in knockout DMEM (Thermo Fisher Scientific) with 15% FBS (Thermo Fisher Scientific), 1000 U/ml LIF (Merck) in presence of 200-300 μg/ml G418 (Thermo Fisher Scientific).

4.2 Mesodermal and myeloid differentiation

For cell differentiation a previously optimized OP9 based protocol was utilized (Senju et al., 2003). ESCs were cultivated on previously seeded feeder layer of OP9 stromal cells in 1:1 ratio using 6 well cell culture plates. An α-MEM (Thermo-Fisher Scientific) based 20% FBS (Thermo-Fisher Scientific), 100 U/ml penicillin and 100 μg/ml streptomycin (Merck) supplemented cell culture medium was used for the 5-day mesodermal differentiation. At day 3 half of the culture medium was refreshed. On 5 day differentiated cells were harvested using 0.25% trypsin-EDTA (Thermo-Fisher Scientific), then further cultivated for 3-6 days using fresh OP9 cell layer (1:5 OP9:ESC derived progenitor ratio) in 6 well cell culture plates in α-MEM medium supplemented with 20% FBS, 50 ng/ml GM-CSF (PeproTech) and 50 μM 2-ME (β-mercaptoethanol; Merck).

For generation of 14 day differentiated cells, 11 day differentiated progenitors were harvested and reseeded onto fresh OP9 layer using the original progenitor-feeder cell ratio and culture conditions.

In some experiments the 5-day differentiated Flk1+ cells were MACS (magnetic cell separation; Miltenyi Biotech) purified and cultured feederless for 24 or 72 hours in α-MEM medium supplemented with 20% FBS, 50 ng/ml GM-CSF, 50 μM 2-ME using 24 well cell culture plates.

EBs were generated as published previously (Kyba et al., 2002) with minor alterations. Culture medium for EB differentiation (EBD) was assembled using IMDM (Thermo-Fisher Scientific) cell culture medium supplemented with 15% FBS, 200 ng/ml iron-saturated transferrin (Sigma), 4.5 mM monothioglycerol (Sigma Aldrich) and 50 ng/ml ascorbic acid (Sigma). ESCs were harvested and seeded in EBD medium using hanging drop formation at a concentration of 100 cells in a 10 μl drop using EBD medium and incubated in inverted bacterial culture dishes. At day 2 EBs were harvested and transferred into 6 cm culture dishes using a low-speed orbital shaker to avoid cell adherence to the culture dish. At day 4 half of the culture medium was refreshed. Cells were harvested either at day 4 or day 6 using 0.25% trypsin-EDTA. In some experiments the harvested cells were further cultured for 3 days on OP9 feeder layer in 6 well cell culture plates (1:5 OP9-progenitor cell ratio) using α-MEM medium supplemented with 20% FBS, 50 ng/ml GM-CSF and 50 μM 2-ME.

4.3 Bone marrow cell isolation and differentiation

Mouse BM cells were isolated from 12-week-old male C57BL/6 or 129S1 animals. The femurs and tibiae were removed, cleaned of connective tissue, and put on ice in DPBS. The ends of each femur and tibia were removed to expose the marrow. For BM-DC differentiation 500,000 BM cells were cultivated for 9 days in RPMI medium containing 10 % FBS (Life Technologies), GM-CSF (50 ng/ml) and 2-ME (50 μM) in 6-well tissue culture plate. Half of the medium was replaced every 3 days. To promote DC activation, medium was replaced with fresh RPMI on day 8 and the cells were exposed with 100 ng/ml LPS.

4.4 Generation of chemically inducible ESC lines

To generate chemically inducible murine ES cell lines targeting vectors (p2lox) containing the ORF of the murine Irf8, Zbtb46, EGFP or bi-cistronic *Zbtb46*-T2A-EGFP were constructed. Coding regions were amplified, using FastStart High Fidelity PCR System (Roche) and Gateway specific primer sequences, from cDNA originated from splenic cell derived samples (Irf8 and Zbtb46) or EGFP containing plasmids. The bi-cistronic vector was generated as the *Zbtb46*-T2A and T2A-EGFP were PCR amplified, then the two fragments were reamplified using Gateway specific attB-containing primers. For the primer sequences see (Table 1.).

The coding sequence of Zbtb46 was amplified using the following Gateway specific primers:
ATTB1-Zbtb46:
GGGGACAAGTTTGTACAAAAAAGCAGGCTGCCACCATGAACAACCGAAAGGAAGATATGGAAA
ATTB2-Zbtb46:
GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAGGAGATCCAGGCAAAGTCTTT
The Zbtb46-T2A-EGFP Gateway compatible DNA fragment was created using the following primers:
ATTB1-Zbtb46:
GGGGACAAGTTTGTACAAAAAAGCAGGCTGCCACCATGAACAACCGAAAGGAAGATATGGAAA
T2A-Zbtb46-rev:
TGGGCCGGGATTTTCCTCCACGTCCCCGCATGTTAGAAGACTTCCCCTGCCCTCGCCGGAGCCGGAGATCCAGGCAAAGTC
T2A-EGFP-for:
GGCTCCGGCGAGGGCAGGGGAAGTCTTCTAACATGCGGGGACGTGGAGGAAAATCCCGGCCCAGTGAGCAAGGGCGAGGAGC
ATTB2-EGFP:
GGGGACCACTTTGTACAAGAAAGCTGGGTCCTTACTTGTACAGCTCGTCCAT
The coding sequence of EGFP was amplified using the following Gateway specific primers:
ATTB1-EGFP:
GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCACCATGGTGAGCAAGGGCGA
ATTB2-EGFP:
GGGGACCACTTTGTACAAGAAAGCTGGGTCCTTACTTGTACAGCTCGTCCAT
The coding sequence of Irf8 was amplified using the following Gateway specific primers:
ATTB1-Irf8:
GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCACCATGTGTGACCGGAACGGC
ATTB2-Irf8:
GGGGACCACTTTGTACAAGAAAGCTGGGTCCTTAGACGGTGATCTGTTGATTTTCTCTA

Table 1. primer sequencing for Gateway specific PCR amplification.

The PCR products were purified with High Pure PCR Product Purification Kit (Roche) and subcloned into pDONR221 plasmid (Thermo Fisher Scientific). Gateway entry vectors were recombined with a p2lox Gateway compatible vector (Bencsik et al., 2016; Iacovino et al., 2014). Final product was purified using Nucleobond Xtra Midi Kit (Macherey-Nagel). To generate chemically inducible cell lines a genetic engineered murine ESC line (ZX1) was utilized as described in (Bencsik et al., 2016; Dandapat et al., 2014). In short, p2lox targeting vector was transfected into ZX1 (genetic background: 129/OlaHsd) ESCs by electroporation (single pulse with 1400 V at 10 msec) using Neon Transfection System (Thermo Fisher Scientific). After the electroporation step cells were seeded onto previously prepared geneticin/neomycin resistant MEF (EmbryoMax, Merck) layer, followed by the chemical selection of the resistant cells for 8 days in knockout DMEM containing 300 μg/ml G418

(geneticin; Sigma Aldrich). On day 8 mouse ESC colonies were gently treated with 0.25% trypsin-EDTA (Sigma Aldrich) and picked up under an inverted phase contrast microscope then cells were reseeded onto freshly prepared MEF feeder layer for further expansion.

4.5 Characterization of the transgenic ESC lines

To evaluate the inducibility of the generated cell clones mouse ESCs were cultured for 2 hours in knockout DMEM (Thermo Fisher Scientific) with 15% FBS (Thermo Fisher Scientific), 1000 U/ml LIF (Merck), 100 U/ml penicillin and 100 μg/ml streptomycin (Merck) and treated with doxycycline 1 μg/ml for 72 hours. Cell culture medium was replaced every 24 hours. After 72 hours cells were pelleted and resuspended in TRI regent (MRC Inc.) and further processed for total RNA isolation. Inducibility of the transgene was quantified using quantitative real time PCR or cells were harvested with 0.25% trypsin-EDTA and analyzed with flow cytometry after intracellular ZBTB46 labeling.

4.6 Maintenance and cryopreservation of MEF and OP9 feeder cells

MEF cells (EmbryoMax, Merck) were maintained in T25 tissue culture flasks using DMEM (Thermo Fisher Scientific) supplemented with 10% FBS (Thermo Fisher Scientific), 100 U/ml penicillin and 100 μg/ml streptomycin (Merck). For freezing, cells were harvested with 0.25% Trypsin-EDTA (Gibco) and cells were frozen using 10% DMSO (dimethyl sulfoxide; SIGMA) containing cell culture medium. OP9 feeder cells were cultured and expanded in T75 tissue culture flasks using α-MEM (Thermo-Fisher Scientific), supplemented with 20% FBS (Thermo-Fisher Scientific), 50 U/mL penicillin and 50 mg/mL streptomycin. For freezing, cells were harvested with 0.05% Trypsin-EDTA (Gibco) and cells were frozen using 10 % DMSO (SIGMA) containing cell culture medium.

4.7 mRNA sequencing

Illumina platform was utilized to perform the mRNA sequencing. Total RNA was extracted using TRI reagent (Molecular Research Center, Inc.), Agilent BioAnalyzer was used for RNA quality control purposes. Library preparation was carried out using Ultra II RNA Sample Prep kit (New England BioLabs) using the manufacturer's recommendations. Sequencing runs were performed on Illumina NextSeq 500 using single-end 75 cycles sequencing. Medical Genomics and Bioinformatics Core Facility of the University of Debrecen performed the library preparation, sequencing and basic bioinformatics. Final sequenced reads were aligned to the mm10 genome assembly (GRCm38), as an output, bam files were generated. The raw data were uploaded to NCBI SRA database under PRJNA647717. Downstream analyses were carried out

using Strand NGS 3.4 (Agilent Technologies). To reveal changing genes, mRNA transcripts were filtered using read density setting 1 (normalized reads per kilobase of transcript per million mapped reads) cut off, and entities in at least 2 of 15 samples showed higher read density than the cut off were selected. Genes were filtered using One way ANOVA (corrected p-value cut-off: 0.05; fold change cut-off: 2; multiple testing correction: Benjamini-Hochberg). The final list of genes was hierarchically clustered and similarity was determined using Squared Euclidean. In order to identify the genes regulated by ZBTB46, transcripts were filtered with read density 1 cut off level on the 6 and 8 day differentiated samples. Entities in at least 2 of 12 samples had higher read density than the cut off were selected. The following setting was used to compare the ZBTB46 induced and non-induced (control) samples from day 6 or day 8: 2-fold change or greater, p-value cut-off was 0.05 using paired T-test. The 6 and 8 day filtered gene lists were combined and hierarchically clustered; similarity was determined by Squared Euclidean.

4.8 RNA isolation and quantitative reverse transcription polymerase gene reaction (RT-PCR)

For total RNA extractions TRI reagent was used. RNA isolation: 0.2 ml chloroform (Merck) per ml of TRI reagent (Molecular Research Center, Inc.) used was added then samples were vortexed for 15 sec and allowed to stand for 10 min at room temperature. The mixture was centrifuged at 12,000 g for 15 min at 4 °C. Upper aqueous phase was transferred into a fresh tube and 0.5 ml of 2-propanol (Merck) per TRI reagent used was added, vortexed and incubated 10 min at room temperature. Then centrifuged at 12,000 g for 10 min at 4 °C. Supernatant was removed, RNA pellet was washed by adding 1 ml of 75 % ethanol (Merck) per 1 ml of TRI reagent used in sample preparation. Sample was vortexed then centrifuged at 12,000 g for 10 min at 4 °C. Supernatant was removed then RNA was air dried for 15 mint and resuspended in molecular biology grade water (Lonza).

The reverse transcriptions were carried out with High-Capacity cDNA RT Kit (Thermo-Fisher Scientific). Quantitative real-time PCR reactions were performed using Roche LC480 or LC96 platform with the following conditions: 1 cycle (for denaturation) at 95°C for 60 sec; 40 cycles at 95°C for 10 sec and 60°C for 30 sec using Taqman (Thermo Fisher Scientific) hydrolysis probes. Assay IDs and gene symbols are listed in Table 2.

Gene symbol	Assay ID
ActB	Mm01205647_g1
Cyp26b1	Mm00558507_m1
Hbb-b1	Mm01611268_g1
Hbb-y	Mm00433936_g1
Irf8	Mm00492570_m1
Ramp3	Mm00840142_m1
Runx3	Mm00490666_m1
Zbtb46	Mm00511327_m1

Table 2. Taqman gene expression assays for RT-PCR.

Comparative threshold cycle method was used to determine the relative gene expression levels normalized to ActB. RT-PCR runs were performed using triplicates and in addition of a control reaction where the cDNA synthesis was performed without reverse transcriptase. Mean ± SD was used to represent the relative gene expression.

4.9 Western blot

Electrophoretic separation of the extracted proteins from 40,000 cells/sample were carried out using 10% polyacrylamide gel then samples were transferred to PVDF membrane (Pall Corporation). Anti-IRF8 polyclonal antibody (ab245607, 2000x dilution, Abcam) and anti-GAPDH monoclonal antibody (AM4300, 2000x dilution, Thermo-Fisher Scientific) were used to probe the membranes.

4.10 Flow cytometry and cell sorting

BD FACS Aria III (BD Biosciences) was used for flow cytometric analyses and cell sorting. 100,000 cells were harvested for surface labeling which were mixed and incubated with 10 µl CD16/32 Fc block solution in DPBS 1:100 for 15 min at 4 °C. Cells were further incubated with 80x diluted antibodies (10 µl blocking buffer + 10 µl diluted ABs) for 30 min at 4 °C. The following anti-mouse antibodies were used for staining: CD45-FITC (30-F11), CD11b-BV711 (M1/70), Flk-1(CD309)-BV421 (Avas 12α1), MHC2-FITC (I-A/I-E; 2G9), CD80-APC (16-10A1), and CD86-APC (GL1) were purchased from BD Biosciences. F4/80–Alexa Fluor 488 (BM8) Ab was obtained from eBioscience (San Diego, CA).

For sorting purposes 1 million cells were harvested and labeled with anti-mouse Flk1 antibody, then 50,000-100,000 Flk1+ cells were sorted. For intracellular ZBTB46 staining Transcription Factor Buffer Set (BD Biosciences) was used per manufacturer's recommendations, staining was carried out using anti-mouse ZBTB46-PE (U4-1374) antibody after surface labeling.

4.11 MACS cell separation

Flk1+ cells were purified using CD309 Microbead Kit (Miltenyi Biotec) according to the manufacturer's instructions with minor modifications. 5 million 5-day differentiated cells were labeled with CD309-Biotin antibody for 10 min at 4 ^{o}C. Then cells were washed with MACS buffer (2% FBS containing DPBS) and labeled with Anti-Biotin microbeads for 15 min at 4 ^{o}C. Cells were washed with MACS buffer and filtered using 30 µm pore size Pre-Separation Filter (Miltenyi Biotec) to remove cell aggregates. Single cell suspension was loaded onto previously prepared MS separation column (Miltenyi Biotec) on a Miltenyi OctoMACS separator, then column was washed 3x with 500 µl MACS buffer, removed from the separator and 1 ml MACS buffer was added and the magnetically labeled cells were eluted.

4.12 Cell cycle and apoptosis assay

Apoptosis detection assay was carried out using FITC Annexin V Apoptosis Detection Kit with propidium iodide (Biolegend) per manufacturer's instructions. In short, harvested cells were resuspended in Annexin V binding buffer and incubated with Annexin V and propidium iodide, respectively.

DNA staining with propidium iodide was utilized to assess the cell distribution in respective cell cycle compartments. Harvested samples were fixed using 70% ethanol then washed and treated with 50 µl RNase (100 µg/ml) and 200 µl propidium iodide (20 µg/ml) solutions. The samples were analyzed on a BD FACS Aria III instrument.

4.13 Hematopoietic colony forming assay

To assess the colony forming potential of the ES derived cells Colony Forming Unit (CFU) assay was utilized using Methocult GF M3434 semisolid medium (Stemcell Technologies). The harvested ESC derived progenitor cells were mixed in DPBS (Dulbecco's phosphate-buffered saline) then seeded into M3434 medium in 3 cm cell culture dishes (1.5 ml/dish). Colonies were identified and counted after 8 days of cultivation with EVOS XL Cell Imaging System (Thermo Fisher Scientific). The following type of blood cell colonies were detected in the ESC derived progenitors: erythroid, GM (granulocyte and macrophage) and GEMM (granulocyte, erythrocyte, macrophage and megakaryocyte) colonies.

4.14 Statistical analysis

Student's t-test (*$p< 0.05$, **$p< 0.01$, ***$p < 0.001$) was used with Excel to assessed and calculate statistical significance.

4.15 ELISA

DuoSet ELISA kits (R&D Systems) were utilized to quantify the production of IL-6, IL-1β and TNF-α cytokines in BM-DC and ES-DC cell cultures. Culture medium was refreshed 24 hours prior to supernatant collection. Preparations and measurements were carried out per the recommendation of the manufacturer. Detection limits for IL-6 and IL1-β: 15.6 pg/ml and for TNF-α: 31.25pg/ml.

4.16 Endocytosis assay

To assess the mannose receptor mediated endocytosis, FITC-dextran 70 (Sigma; average molecular mass 70 kDa) was used. Immature BM-DCs or ES-DCs were incubated with 1mg/ml FITC-dextran ($1.5x10^5$ DCs/well in 24 well cell culture plate) at 37 °C for 60 min then quantified by flow cytometry. Phagocytic capacity of the ES-DCs and BM-DCs was determined by the cellular internalization of latex beads (Sigma; carboxylate modified, mean diameter 1 μm. Immature ES-DCs or BM-DCs were mixed with latex beads (1.5×10^5 DCs + 1.2×10^6 latex beads/well in a 24 well-plate) at 37°C for 120 min (control was kept at 0°C), then latex bead internalization quantified with flow cytometry.

4.17 DC migration assay

Transwell migration assays were utilized for the assessment of the CCL19 (C-C motif chemokine ligand 19) and CCL21 (C-C motif chemokine ligand 21) chemokine induced DC migration. 24 well plates with 5 μm pore size (Corning-Sigma) were used. The lower chambers were loaded with 600 μl RPMI-1640 medium in presence or absence of chemokines. Chemokine concentration was set to: CCL19 (500 ng/ml; R&D Systems), CCL21 (500 ng/ml; R&D Systems). 2×10^5 LPS primed BM-DCs or ES-DCs were transferred into the Transwell inserts (volume 100 μl) and incubated for 4 hours at 37 °C. Then, the cells from the lower chambers were collected and counted using flow cytometry (samples acquired for 3 minutes at a constant flow rate).

4.18 T cell proliferation analysis

T cells were isolated from spleens obtained from 12-week-old male BALB/c mice by the Miltenyi Pan T cell isolation kit II (Miltenyi Biotec). For allogeneic MLR (mixed leukocyte reaction) 10^3 or 10^4 ES-DCs (genetic background: 129/OlaHsd) as stimulator were co-cultured with 10^5 T cells using 96-well round-bottomed culture plates for 5 days. BrdU (5-bromo-2'-deoxyuridine) was added upon the last 12 hours of the culture. At the end, half of the cells were

centrifuged and the BrdU incorporation was assessed with a Cell proliferation Assay kit (Merck) according to the manufacturer's recommendations.

5. Results

5.1 Enhanced DC maturation by RUNX3

To improve our understanding on ES-DC development, my colleagues examined the efficiency of DC generation from mouse ESCs and analyzed the phenotype of the obtained antigen presenting cells. Interestingly, two DC maturation markers (MHCII and CD80) were poorly expressed upon LPS administration in ES-DCs (less than 25% MHCII/CD80+ cells were detected). In contrast, more than 50% percent of MHCII/CD80+ cells were obtained from BM-DCs. In addition, they observed that the LPS-activated ES-DCs showed a heterogeneous CD86 expression, in contrast, most of the LPS-treated BM-DCs were CD86+ (Takacs et al., 2017). Of note, both the CD80 and CD86 are DC specific maturation markers which can provide costimulatory signals necessary for T cell activation and survival (Somoza and Lanier, 1995). These findings revealed that ES-DCs represent a distinct myeloid cell type with a limited maturation capacity. To further characterize these immune cells the transcript levels of numerous transcription factors in ES-DCs were directly compared to BM-DCs. Erika Takács' analysis revealed that three genes (*Spi-B, Irf4* and *Runx3*) exhibited a significantly lower expression in ES-DCs versus BM-DCs. In line with the transcript profile, detectable level of RUNX3 protein was measured from BM-DCs, in contrast, this transcription factor was barely detected from ES-DCs. This altered gene expression prompted us to test the effects of the RUNX3 transcription factor in ES-DCs and their progenitors using chemically inducible transgenic ESCs. Of note, my colleagues have previously generated *Runx3* and *Spi-B* (Spi-1/PU.1 related transcription factor) expressing ESC clones by Cre-mediated recombination (Takacs et al., 2017).

In this study first, we tested the effect of the transgenic *Runx3* on the later stage of the *ex vivo* DC differentiation. RUNX3 inducible ESC clones (C2 and C4) were differentiated into ES-DCs for 19 days as described in the Methods section. This transcription factor was overexpressed between day 5 and day 19 and cells were also treated with LPS at day 18 to generate mature DCs and to boost their activation. For these experiments we purified the CD45+ cells at day 10 by FACS and these cells were further cultured for 9 days. This step was carried out to ensure the RUNX3 expressing hematopoietic cells were collected without major loss of cells as a tendency of their attachment to the OP9 feeder lays was previously observed.

To characterize the maturation capacity of our ESC derived terminally differentiated DCs, we assessed their MHCII and CD80 expression pattern at day 19. In line with our expectations an elevated MHCII/CD80 double positive subpopulation was observed as response to the LPS treatment. Strikingly, upon introduction of *Runx3* (doxycycline treated cells) elevated number of MHCII/CD80+ cells were observed and also a unique MHCII+ subset was emerged in our RUNX3 instructed cells (**Figure 4**). Although these cells still showed lower MHCII/CD80 expression compared to the BM-DCs (**Figure 4B**), our findings indicate the enhanced maturation potential by RUNX3.

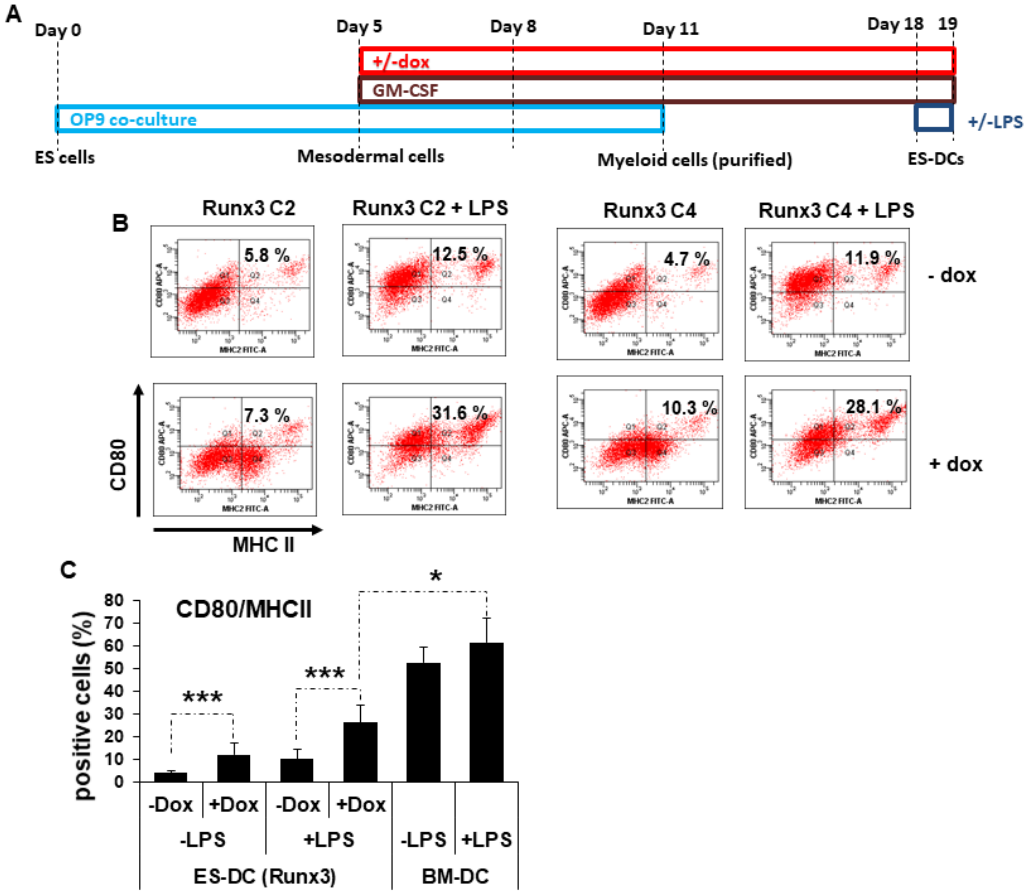

Figure 4. Elevated level of MHCII expression by Runx3 in 19 day differentiated ES-DCs. (A) Schematic representation of the RUNX3 driven ES-DC differentiation. (B) Genetically engineered ES derived cells were induced with 1 µg/ml doxycycline (+ dox) and differentiated as outlined in the Materials and Methods. At day 18 a fraction of the cells was also treated with LPS (100 ng/ml) as marked. Flow cytometry was utilized to evaluate the MHCII and CD80 expression on the cell surface at day 19. Figure shows the data of 2 representative Runx3 inducible cell lines (C2, C4). (C) Runx3 inducible cells were cultured as described in (B). BM-DCs were cultured for 9 days, at day 8 cells were primed with LPS (100 ng/ml) MHCII and CD80 assessments were performed after 9 days of differentiation. Eight (ES-DC, both C2 and C4 clones) or 6 (BM-DC) independent experiments were performed to determine the average ratio and SD of the MHCII/CD80 double positive subpopulation. *p< 0.05, ***p < 0.001.

Next, we also tried to analyze the effects of the transgenic Irf4 and Spi-B proteins. My colleagues previously found that, much less CD45+ myeloid cells were obtained upon the enforced expression of Irf4 (Takacs et al., 2017) suggesting that this regulatory protein elicited a detrimental effect on the early stage of ES-DC development. Consistent with this finding only a few live cells were detected at day 19 from the Irf4 inducible, sorted blood cells upon doxycycline treatment (data not shown). The poor yield precluded the detailed flow cytometric analysis and this finding strongly suggest that Irf4 provokes a general inhibitory effect during ES-DC differentiation. In contrast to the *Runx3* expression, upon Spi-B induction no change was detected in our MHCII/CD80 maturation markers (**Figure 5**).

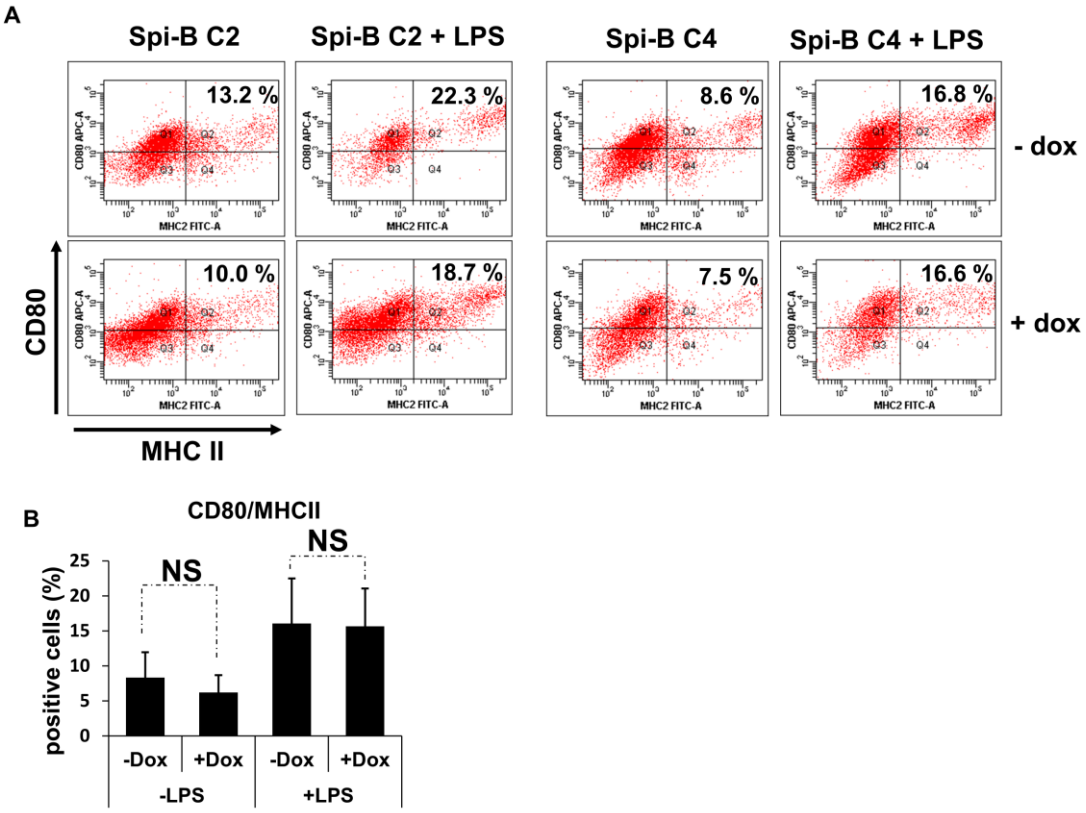

Figure 5. Overexpression of Spi-B does not alter the ES-DC maturation process. (A) Spi-B was induced in nineteen-day differentiated cells using 1 µg/ml doxycycline (+ dox) and at day 18 cells were exposed to LPS (100 ng/ml) as indicated. MCHII and CD80 cell surface profile was characterized with flow cytometry at day 19 using 2 inducible Spi-B cell lines (C2 and C4). (B) Spi-B instructed cells were cultured as described in (A), calculations of the MCHII/CD80 ratio as well as the SD were based on 6 independent experiments. Statistical significance was analyzed using Student's t-test (NS: p > 0.05)

These data indicate that the ectopic expression of *Runx3* can selectively promotes the ES-DC maturation. To further examine the maturation capacity of our RUNX3 instructed DCs we

tested their CD86 expression level. Consistent to the enhanced expression of MHCII/CD80, elevated number of CD86+ cells were also detected in our LPS induced, RUNX3 primed cells (**Figure 6**). We also evaluated the expression of the F4/80 macrophage specific marker which failed to show any alterations upon LPS treatment or *Runx3* induction. These results altogether proved that RUNX3 positively modulates the activation and maturation potential of ESC derived DCs, *ex vivo*.

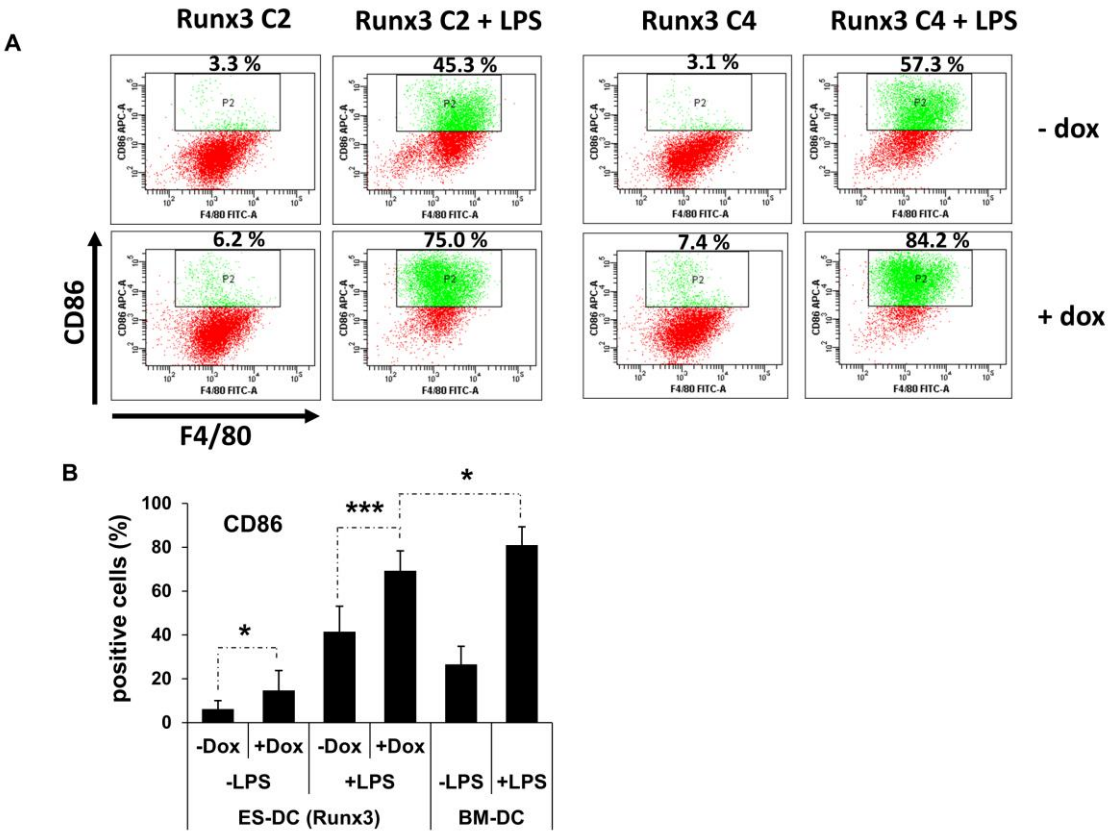

Figure 6. Improved Runx3 driven maturation capacity. (A) Cells were cultured for 19 days and Runx3 was induced using 1 µg/ml doxycycline (+ dox) as indicated. Cells were primed with LPS (100 ng/ml) at day 18, as marked. CD86 and F4/80 expressions were determined at day 19 using flow cytometry. Representative data were derived from C2 and C4 Runx3 inducible ES clones. (B) Runx3 driven ES-DCs were cultured as described in (A), at day 8 BM-DCs were primed with LPS (100 ng/ml) MHCII and flow cytometric assessments were performed after 9 days of differentiation. Eight (ES-DC) or 6 (BM-DC) independent experiments were performed to determine the average ratio and SD of the CD86+ subpopulation. *p< 0.05, ***p < 0.001.

5.2 Improved migratory capacity and T cell activation potential of ES-DCs by RUNX3

Next, we tested if RUNX3 can modulate the functional attributes of our ESC derived DCs. FITC-dextran internalization was measured to assess the receptor mediated endocytic capacity of our cells and we also utilized latex bead uptake to test the phagocytic capacity of these cells.

The result of these analyses showed the immature ESC derived DCs were effectively performed the latex bead uptake, although no further advancement of this process was observed upon *Runx3* introduction (**Figure 7A**). Overexpression of *Runx3* in ESC derived DCs however negatively impacted their performance to engulf FITC-dextran and a moderate FITC-dextran engulfment was also observed in BM derived DCs compared to control ESC derived DCs (**Figure 7A**).

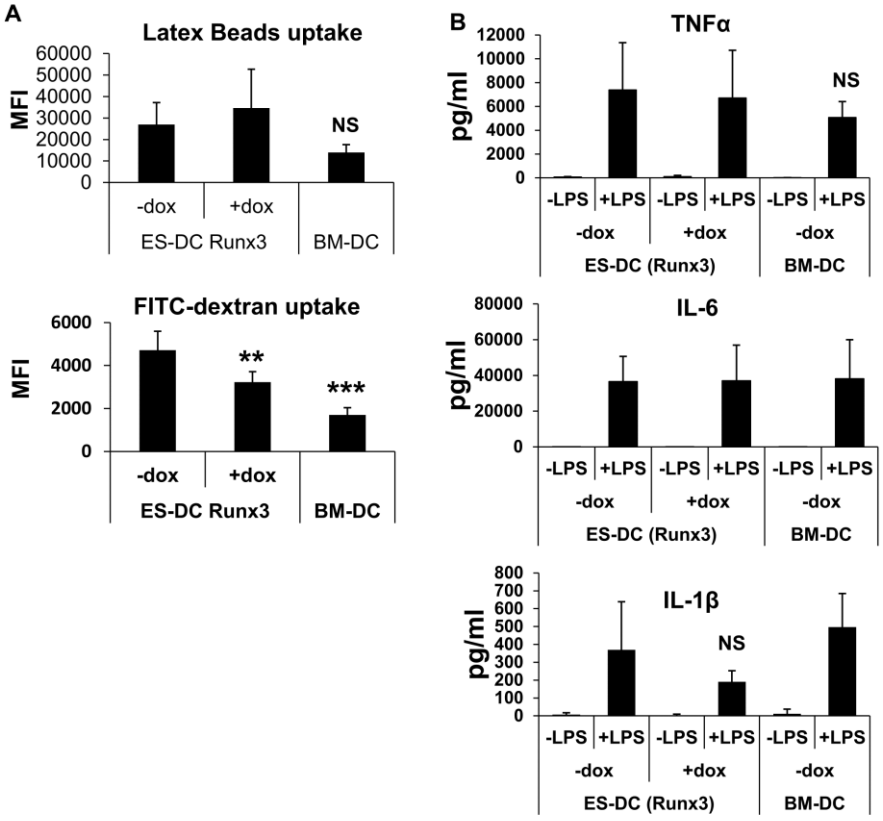

Figure 7. Phagocytic capacity and cytokine production profile of the RUNX3-primed ES-DCs. (A) Measurement of latex beads and FITC-dextran uptake was used to characterize the endocytic potential of Runx3 inducible immature ES-DCs and BM-DCs. In ES-DCs the transgenic Runx3 was primed using 1 µg/ml doxycycline (+ dox), flow cytometry was utilized to assess the mean fluorescence intensity (MFI), data of 3 independent experiments were used to determine the MFI and SD values. (B) TNF-α, IL-1β and IL-6 cytokine concentrations from the supernatants of our cell cultures were measured with ELISA. In ES-DCs the transgenic Runx3 was primed using 1 µg/ml doxycycline (+ dox). Supernatants were obtained after 24 hours of the LPS (100 ng/ml) treatment as indicated and in parallel, from the control cell culture. Data of 8 independent experiments were used to calculate the average of the cytokine concentrations and SD. **p < 0.01, ***p < 0.001.

These results showed the endocytic and phagocytic capacity of our immature, ES-DCs and demonstrated these processes were either not affected or even suppressed by the introduction

of *Runx3*. To determine the cytokine production of our cells we utilized ELISA measurements. Notably, in LPS induced ES-DCs an elevated TNF-α and IL-6 production was detected together with IL1-β (**Figure 7B**). Interestingly no change was detected in the production of these cytokines in presence or absence of RUNX3. The detected cytokine expressions in ES-DCs were similar to the detected levels in BM-DCs derived supernatants. In the light of these results, we can conclude that our ESC derived DCs have a great potential to proinflammatory cytokine production, and even though the introduction of our transgene enhanced the expression several maturation markers, the production of proinflammatory cytokines was not modified by RUNX3.

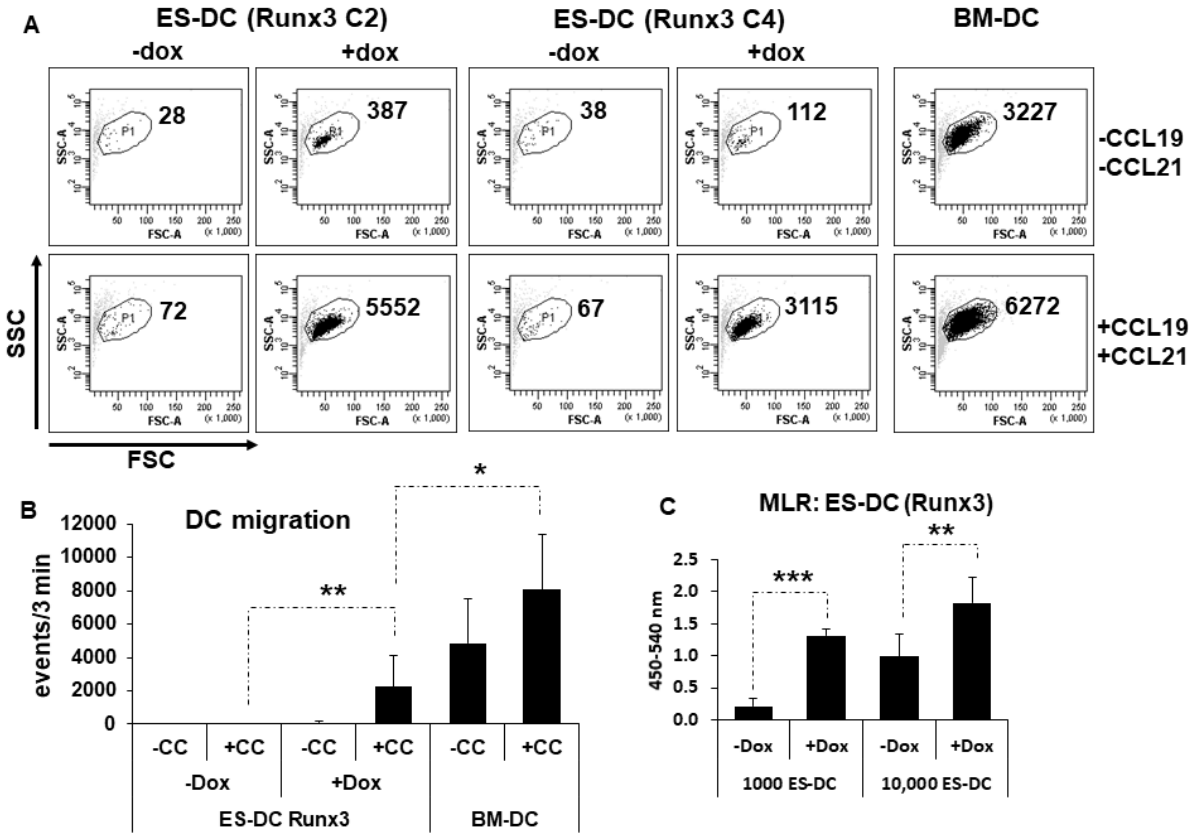

Figure 8. Runx3 boosted migratory and T cell priming potential. (A) Mature ES-DCs and BM-DCs were tested for their migratory potential in presence or absence of CCL19 and CCL21 using Transwell migration assays. In ES-DCs the transgenic Runx3 was primed using 1 µg/ml doxycycline (+ dox), then cells were counted by flow cytometry. The corresponding event count of 3 minutes were represented on dot plots using forward scatter (x axis) and side scatter (y axis). (B) The calculations of the averages and SD of the migration rate were based on 6 ES-DC and 3 BM-DC independent experiments and the migration potential was assessed in presence (+CC) or absence (-CC) of CCL19 and CCL21. (C) T cell priming potential was evaluated using MLR. Nineteen-day differentiated Runx3 instructed, LPS induced ES-DCs were used for T cell stimulation. 1000 or 10000 ES-DCs as stimulator were co-cultured with 10^5 T cells using 96-well round-bottomed culture plates for 5 days. Runx3 was induced with 1 µg/ml doxycycline (+ dox) as indicated. The T cell activation capacity was tested using BrdU Cell Proliferation Assay. The calculations of the corrected absorbance (450-540nm) and SD values were based on 4 independent experiments (both the C2 and C4 Runx3 clones were tested). *p < 0.05, **p < 0.01, ***p < 0.001.

Next, we tested the CCL19/CCL21 based migratory potential in our cells utilizing Transwell migration assay. Cells were incubated for 4 hours in the transwells then the migrated cells were collected and counted with flow cytometry. Our data showed the migratory potential of the RUNX3 driven ES-DCs was adequate, however our ES-DCs without RUNX3 showed an impaired capacity even in company with CCL19 and CCL21 (**Figure 8**). As a benchmark we

also evaluated the migration capacity of BM derived DCs. Strikingly these cells showed higher level migratory potential even without the addition of the cytokines mentioned above. Our results demonstrated the elevated migration potential of our RUNX3 driven DCs, however the level of this cell function was still below the benchmark level set by BM-DCs.

As a last step we tested the T cell activation potential of the *Runx3* transgenic ES-DCs by MLR reactions. In these experiments the ES-DCs were co-cultured with T cells possessed different genetic background (allogeneic stimulation). In line with the improved maturation potential, we detected an enhanced T cell proliferation rate as response to *Runx3* induction in ES-DCs (**Figure 8C**). In conclusion, our results suggest that RUNX3 enhances the migration potential and T cell activation capability of the ESC derived antigen presenting cells. Our analysis of the phenotype supported by the evaluation of the DC function showed that *Runx3* overexpression enhances the immunogenic properties of our ESC derived DCs. Our observation that compared to BM-DCs only partial reconstitution of DC differentiation could be achieved prompted us to examine the function of other transcription factors.

5.3 ZBTB46 dependent impaired myeloid cell differentiation

Our colleagues have compared the mRNA expression level of 17 different DC specific transcription factors in ES derived *ex vivo* generated DCs (Takacs et al., 2017). Some of these factors showed highly elevated mRNA expression, other transcripts were however poorly represented. Similar to RUNX3, the cDC marker ZBTB46 was barely expressed in ES-DCs and their progenitors (Takacs et al., 2017). Therefore, in this study we also aimed to reveal the role of this transcription factor in the ES-DC differentiation using a similar gain of function approach as we applied for the RUNX3 study. Chemically inducible *Zbtb46* expressing ESCs were constructed in a genetically engineered system and several stable *Zbtb46* expressing clones were established. Two ESC clones (C2 and C4) were selected, further expanded and characterized. Our experimental data shows the mRNA expression level of *Zbtb46* highly elevated after 3 days of doxycycline treatment and ZBTB46 was also successfully detected in the protein level in 95% of the induced cells (**Figure 9**). These together proved the ZBTB46 transcription factor can be uniformly induced in our ESC based transgenic system.

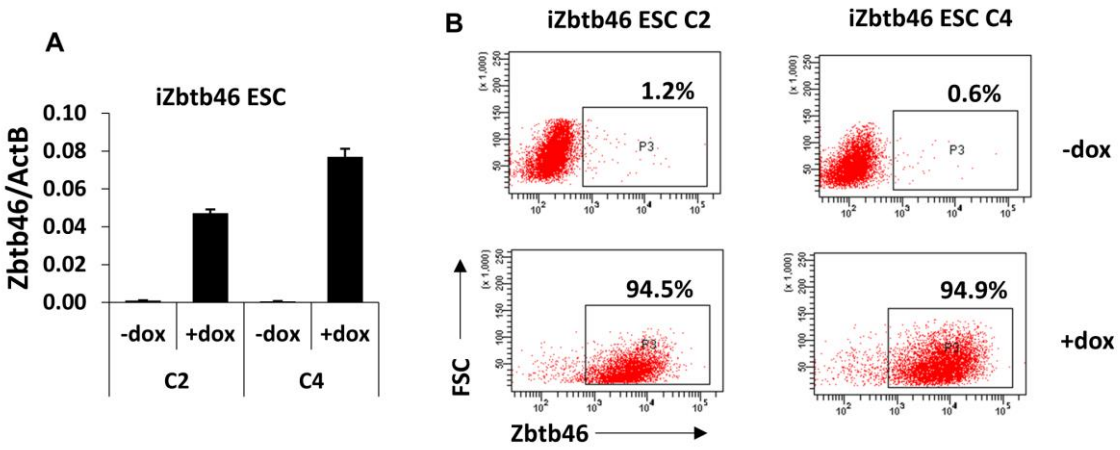

Figure 9. Evaluation of the transgenic ZBTB46-inducible ESC clones (C2 and C4). (A) mRNA expression of Zbtb46 was determined by quantitative RT-PCR. Transgenic cells were induced with doxycycline for 48 h (+dox). (B) Single cell level quantification of the ZBTB46 protein with intracellular flow cytometry using the ESC clones outlined in part (A) after 48 h induction of the transgenic Zbtb46. (FSC, forward scatter).

The validation process of the newly generated clones was followed by the assessment of this factor in *ex vivo* ESC differentiation. To obtain ES-DC progenitors we utilized an OP9 cell-based culture method which was previously described (Senju et al., 2003). Overexpression of the transgene started at day 5 and the progenitor cells were harvested in 3 different timepoints: at days 8, day 11 or day 14 (**Figure 10A**). Interestingly the CD45+/CD11b+ fraction was greatly diminished by ZBTB46 in 8 day differentiated state of our ES derived cells. It is worth mentioning that CD45 is a receptor-linked protein tyrosine phosphatase that is expressed on all leucocytes including myeloid blood cells (Hermiston et al., 2003). In contrast, CD11b is a myeloid cell specific marker. This result suggests the strong repressive effect of ZBTB46 on myeloid commitment. The 11 day differentiated progenitors also showed a lowered myeloid potential upon ZBTB46 however this effect was slightly moderate. The impaired myeloid cell formation was also observed at day 14 (**Figure 10**).

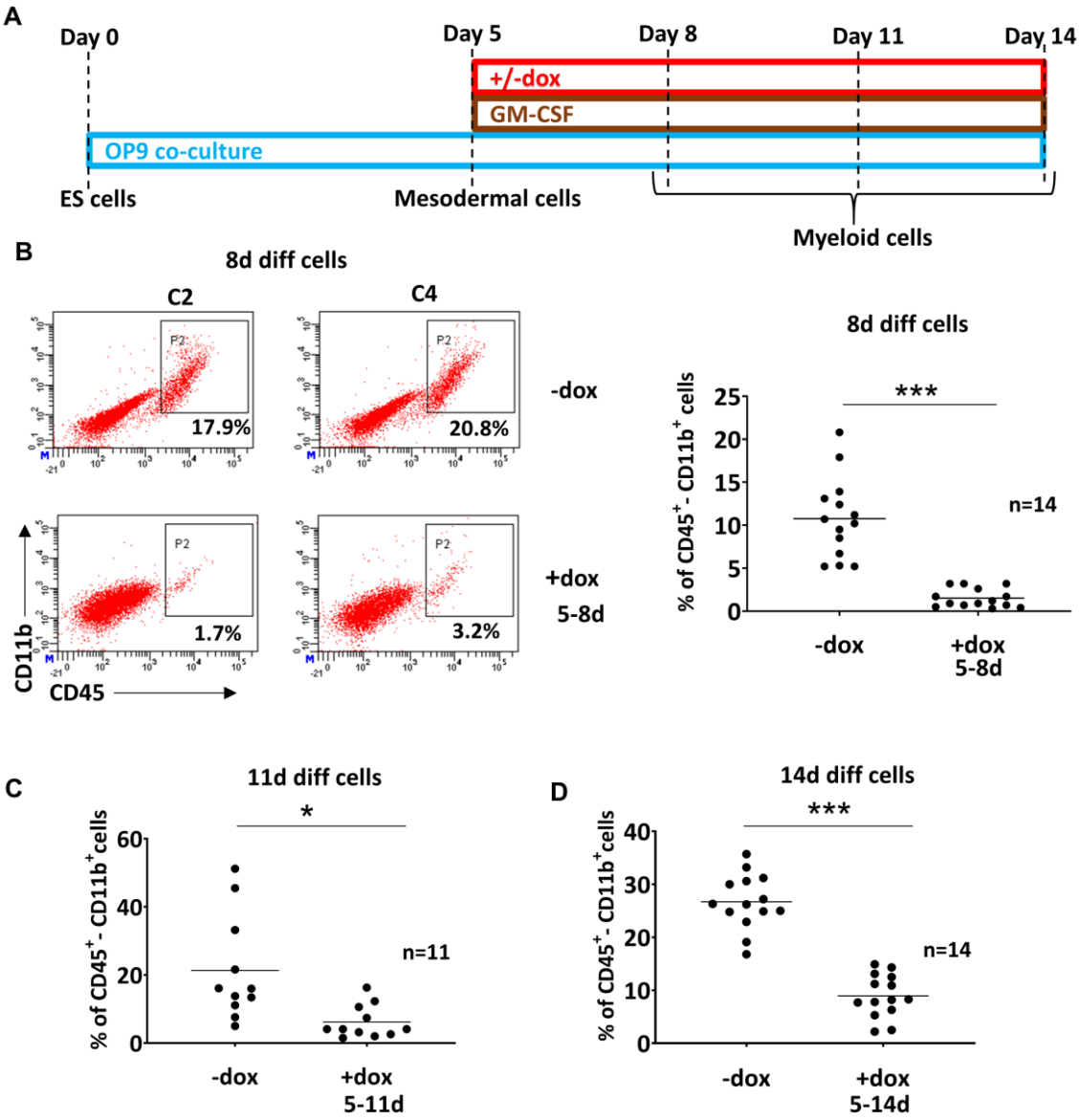

Figure 10. ZBTB46 impacted myeloid cell differentiation. (A) Schematic representation of the ZBTB46 driven ES cell fate determination. Flow cytometric assessments were performed at day 8, 11 or 14. (B) Figure shows the CD45+/CD11b+ distribution in ESC derived 8-day differentiated cells. (C, D) Statistical representation of CD45+/CD11b+ subset at (C) day 11 and (D) at day 14 of the cell differentiation. Zbtb46 was induced during the indicated period of time.

We also tested the effect of ZBTB46 in progenitors derived from EBs. These three-dimensional cell aggregates were grown for 6 days and the disaggregated EB derived progenitors were further cultured with OP9 cells for 3 days. Similar to the monolayer differentiation, the EB derived cells poorly converted to CD11b+/CD45+ myeloid blood cells in the presence of ZBTB46 (**Figure 11**). These results further support the general myelosuppressive role of ZBTB46 in our murine ES derived transgenic system.

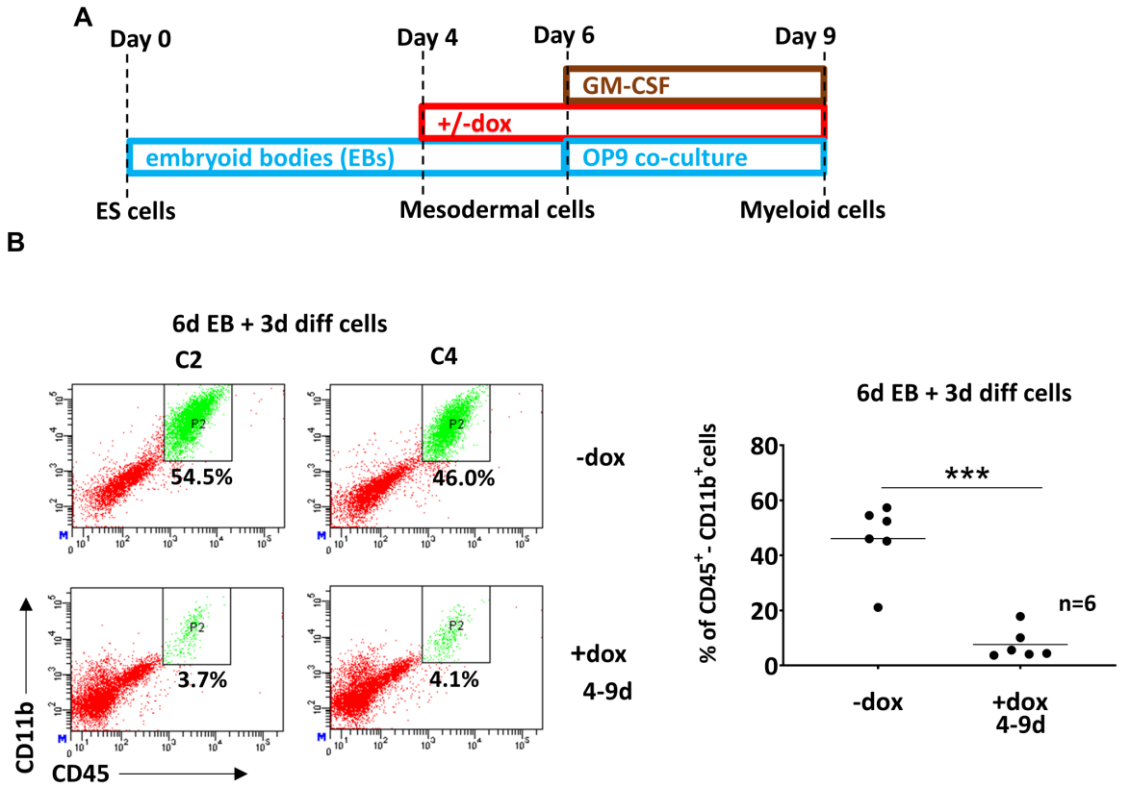

Figure 11. Negative effect of ZBTB46 on myeloid cell production in EB-derived progenitors. A, Experimental scheme of the blood cell differentiation via EB formation. B, Representative flow cytometric data and statistics show the % of the CD45/CD11b double positive cells testing the 9-day differentiated cells.

5.4 Inhibition of myeloid commitment in *Zbtb46* expressing ES derived cells

Although an overall inhibitory effect was observed in response to the introduction of *Zbtb46* a detectable fraction of CD45+ cells were still observed at day 11 and 14 in the doxycycline treated samples, indicating a remaining potential to differentiate into myeloid cells. We further examined the myeloid commitment of our ES derived cells to reveal and understand whether an elongated myeloid cell development occurred driven by ZBTB46 or the transgene was not expressed in a specific subset of our transgenic cells. In ESCs a uniform transgene expression was observed (**Figure 9**B), nonetheless the transgene can be silenced in other developmental stages. To evaluate this possibility, we determined the intracellular protein level of ZBTB46 together with CD11b on the cell surface at day 11 and day 14 (**Figure 12**). Interestingly only 40-50% of the cells were ZBTB46+ at day 11 and we detected an even lower, 10-15% ZBTB46 positivity in the 14 day differentiated cells. Important to mention, most CD11b+ cells were Zbtb46 negative, indicating a greatly impaired myeloid commitment of our *Zbtb46* expressing

ES derived progenitors. To support this finding, we engineered a bi-cistronic transgenic system where *Zbtb46* and EGFP can be chemically induced at the same time using doxycycline. In addition, we generated an inducible EGFP expressing ESC cell line to serve as an experimental control. In line with the intracellular ZBTB46 labeling most of the CD11b+ cells failed to express EGFP in case of the bi-cistronic cells (Zbtb46-EGFP), however the GFP+/GFP- ratio in the CD11b+ subpopulation was elevated in absence of *Zbtb46* (**Figure 12**).

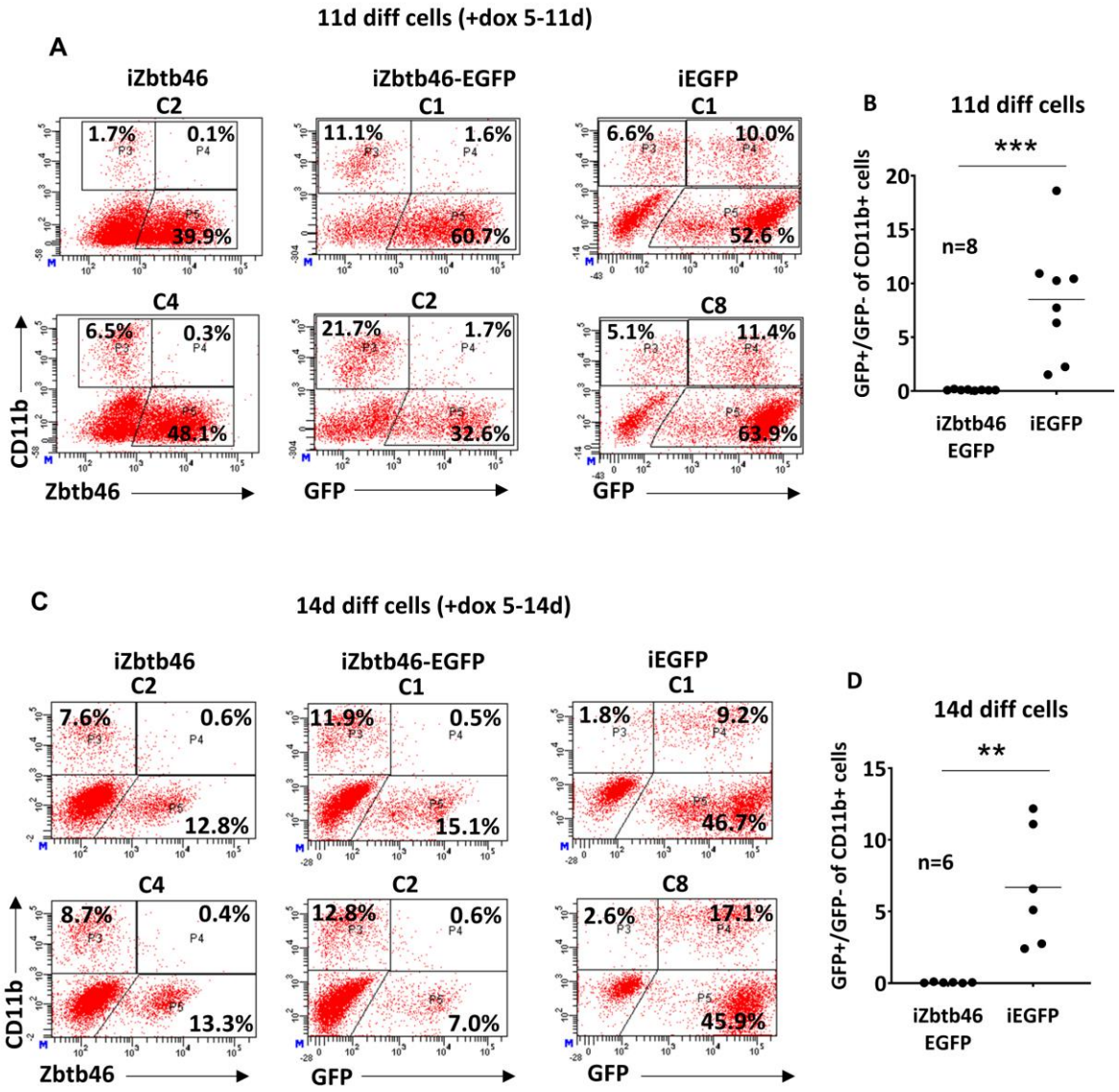

Figure 12. ZBTB46/EGFP expression in ex vivo differentiated cells. (A) Quantification of ZBTB46 protein at day 11 using intracellular flow cytometry in Zbtb46 driven C2 and C4 ESC clones. Cells were labeled with CD11b antibody prior to intracellular staining. Quantification of GFP and CD11b in Zbtb46/EGFP inducible C1 and C2 clones are also represented. Transgene was induced between day 5 and day 11. (B) Statistical representation of GFP+/GFP- distribution in transgenic CD11b+ cells at day 11. (C) Zbtb46 and GFP expression of ESC clones outlined in (A) at day 14 of the differentiation. Transgene was induced between day 5 and day 14. (D) Statistical representation of GFP+/GFP- distribution in transgenic CD11b+ cells of Zbtb46/EGFP or EGFP ESC clones at day 14.

Altogether our results uncovered the suppressive effect of ZBTB46 in the ESC derived myeloid development. It was also shown that the applied transgenic system has restricted potential for the assessment of later stages of the ESC based DC development as most cells were ZBTB46- at day 14. We also tried to test the inducibility of our transgene at a later timepoint of the

development, however we were not able to induce *Zbtb46* in ESC derived 11 day differentiated cells (data not shown). This phenomenon is not a transgene dependent as the EGFP was also barely induced upon doxycycline treatment started at day 11 (data not shown). In conclusion our results revealed our transgenic system is well suitable to assess the effect of ZBTB46 in the early ESC-mesoderm and hematopoietic developmental stages, but not ideal to test the transgenic effect of ZBTB46 in myeloid lineage committed cells.

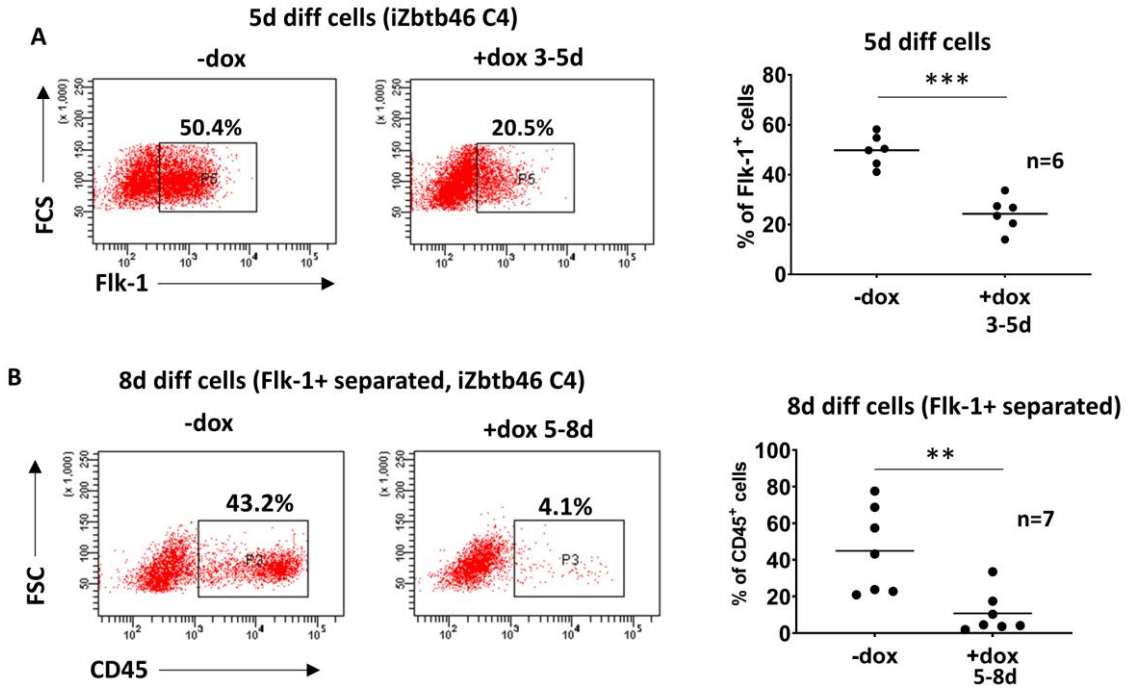

Figure 13. Impaired mesodermal and myeloid transition by ZBTB46 testing the Zbtb46 inducible (iZbtb46) C4 cells. (A) Flow cytometric images show the proportion of Flk1+ subpopulation in Zbtb46 instructed 5 day differentiated ESC clones (FSC, forward scatter). (B) Repressed myeloid cell commitment by ZBTB46 in Flk1+ purified cells. Images show the CD45+ ratio of ZBTB46 driven progenitors derived from Flk1+ cells at day 8. Flk1+ cells were purified at day 5 and transgene was upregulated between day 5 and day 8.

5.5 Myeloid lineage suppression by ZBTB46 in Flk1+ mesodermal cells

Next, we assessed the early mesodermal development of our *Zbtb46* expressing, transgenic cell lines between day 3-5 of the differentiation. Our results showed introduction of *Zbtb46* resulted in an impaired Flk1+ cell formation at day 5 (**Figure 13A**) demonstrating the negative impact of this transcription factor on not only the myelopoiesis but also the ESC-mesoderm transition. This observation opened the question if the limited capacity to give rise myeloid cells is a direct effect of the impaired mesodermal cell development. Cells in pre-mesodermal state can possibly exist among our 5 day differentiated cells which cannot transition into Flk1+ myeloid precursors. To test these possibilities 5 day differentiated Flk1+ cells were purified using

MACS cell separation and differentiated for 3 days feederless with the overexpression of our transgenic *Zbtb46*. Strikingly, similar to the cells without magnetic separation the ratio of the CD45 expressing cells were greatly reduced in presence of ZBTB46 in our magnetically separated Flk1+ myeloid precursors (**Figure 13B**). It was also concluded the ZBTB46 driven suppression can also be observed in feederless cell cultures, without the use of OP9 cells.

5.6 ZBTB46 induced the cell cycle changes in ESCs

Along with the previously uncovered attributes of the ZBTB46 driven ES cell differentiation an altered cell number was also observed as a result of this transcription factor. Lower number of cells were assessed from our differentiated cells between day 0-5 or at day 8 and day 11. This phenomenon was even observed in ESC cultures upon introduction of *Zbtb46* (**Figure 14A**).

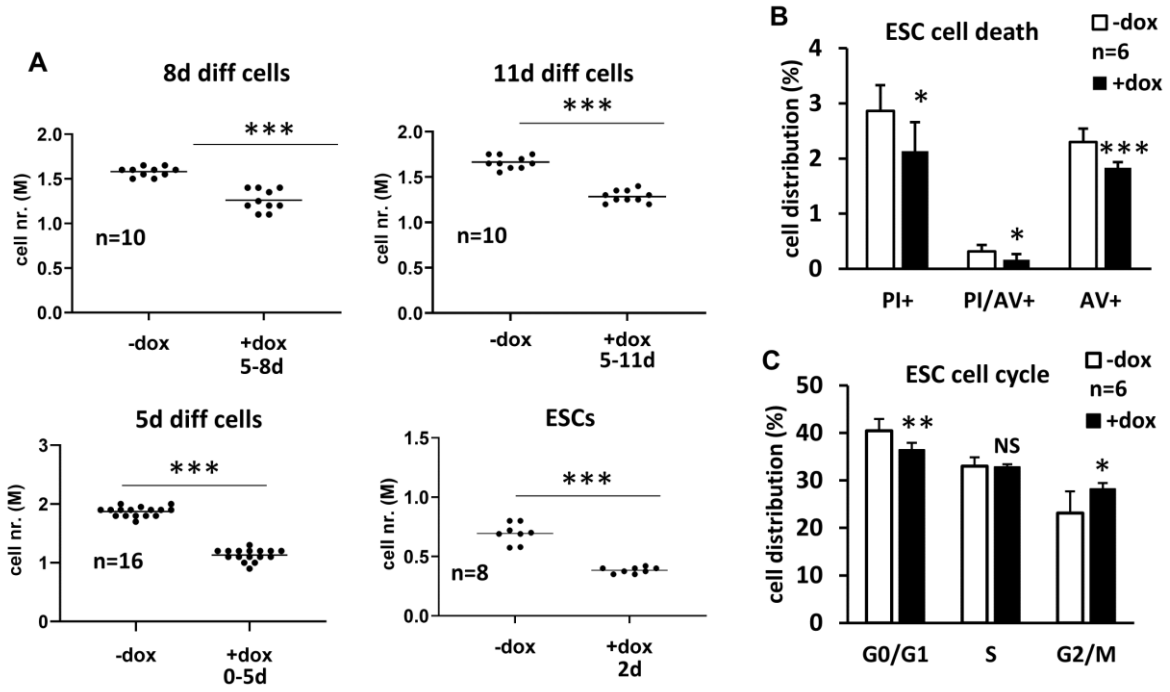

Figure 14. Cell death, cell number and cell cycle distribution modulated by ZBTB46. (A) Images show ZBTB46 impacted cell counts in different stages of cell differentiation. Absolute cell counts of a well from a 6-well cell culture plate are represented, transgene was induced as indicated. (B) Quantification of ZBTB46 modified cell death profile of ZBTB46 inducible clone 4 (C4) ESCs. Cells were labeled with Annexin V (AV) and propidium iodide (PI) then acquired in flow cytometer, Zbtb46 was induced for 72 h. (C) Assessment of cell cycle phases in ZBTB46 inducible C4 ESCs. Cells were treated with PI after permeabilization to assess the distribution of cell cycle compartments with flow cytometry. Cells were treated for 72 h with doxycycline prior to testing.

These results indicated that ZBTB46 impacts the cell viability or the proliferation capacity of our undifferentiated cells or ESC derived progenitors. To test these possibilities, we determined the rate of cell death in our transgene driven system and also assessed the cell cycle profile to uncover any possible changes indicated by ZBTB46. Strikingly, we observed lower propidium iodide and annexin V positivity in ZBTB46 instructed cells compared to the control (without *Zbtb46* induction), non-differentiated ESCs (**Figure 14A**) indicating neither viability nor the formation of apoptotic cells can be accounted for the decreased cell number. Moreover, an altered cell cycle profile was observed upon introduction of *Zbtb46*, the ratio of G0/G1 cells was lowered however the fraction of cells in G2/M phase was elevated in ZBTB46 driven ESCs. In the light of our result, we concluded this delay in the cell cycle process can be accounted for the negative impact on cell proliferation.

5.7 Gene expression pattern altered by ZBTB46

In order to reveal the ZBTB46 driven gene expression changes we optimized our protocol to achieve homogenous cell populations. Five day differentiated and MACS purified Flk1+ cells were cultured feederless in presence or absence of doxycycline. We harvested the cells at 24 hours to observe the early effect of ZBTB46 and sampling was also taken place after 72 hours (**Figure 15A**). Genome wide gene expression analysis was performed using RNA sequencing to test 3 biological replicates per experimental condition (total of 15 samples). As first we observed the expression profile of *Zbtb46* and accordance with our expectations the transcript levels were greatly elevated in the doxycycline treated samples (**Figure 15B**).

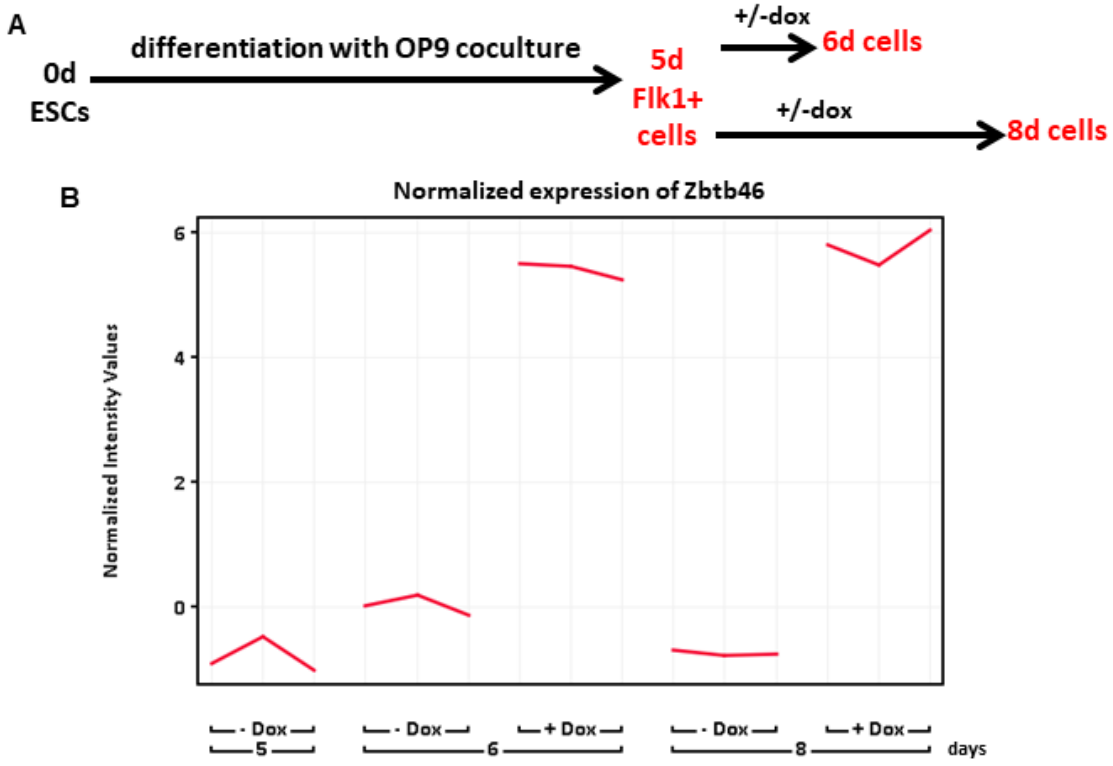

Figure 15. Transcript profile of ZBTB46 instructed cell development. A, Schematic representation shows specific stages of cell differentiation where samples were collected for gene expression profiling. B, Zbtb46 expression pattern measured by RNA-Seq. Three biological replicates were used.

Next, global gene expression analysis was performed using principal component analysis which showed the 5 day differentiated Flk1+ cells has a unique gene expression pattern compared to the 6 or 8 day differentiated cells. This might be an indication of the moderate effect to the global expression signature by doxycycline (*Zbtb46* induction). In addition, ANOVA test was used on the preselected genes to determine the differential gene expression within the tested 5 experimental conditions. Our analysis revealed 3030 changing genes of the 15371 expressed genes showing a dynamic profile at this differentiation stage. We observed a specific set of genes with increased expression at day 8, however interestingly most of these high expressors were downregulated by ZBTB46 (**Figure 16**). This result was in line with the negative phenotypic effect of the transgene after 3 days of doxycycline administration.

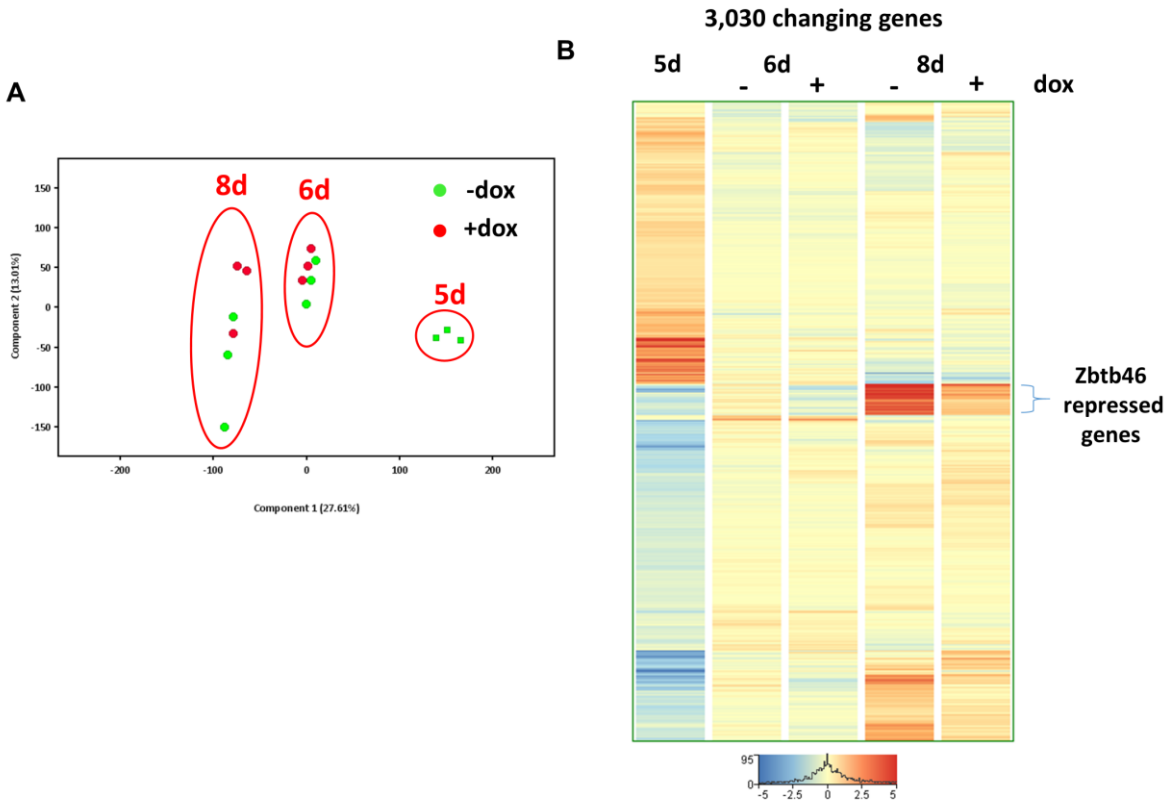

Figure 16. Gene expression profile of the ZBTB46 activated cells. A, Principle component analysis for visualization of the alterations of stage and transgene (doxycycline) dependency. B, Hierarchical clustering of 3030 changing genes. This gene set contains a well-defined ZBTB46-repressed gene cluster at day 8.

We further analyzed the transcripts repressed by ZBTB46 using a new approach including only the transgenic effect of ZBTB46 in our ESC derived 6 and 8 day differentiated cells. Our analysis showed decreased expression of 726 transcripts in 6 or 8 day differentiated cells instructed by ZBTB46 and more interestingly as our cluster analysis pointed out several of these were involved in both day 6 and day 8 (**Figure 17A**). To determine the gene ontology (GO) groups among these genes, gene set enrichment analysis was performed. Notably, GO categories associated with immune cell were highly represented over other classes (data not shown). For example, the category "immune system process" include 148 transcripts involving Itgal (DC11a), Itgam (CD11b) and Irf8 (**Figure 17A**). Strikingly all these genes were repressed even more prominently at day 8 suggesting several genes associated with immune and myeloid cell development are negatively regulated in those cells instructed by ZBTB46. Validation of the impaired expression of Irf8 at day 8 was carried out using quantitative PCR and Western blot with independent sets of samples (**Figure 17B and C**). In line with our flow cytometric assessments, ZBTB46 greatly repressed the majority of genes involved in the myelopoiesis and

our genome-wide transcript analysis served as evidence to the suppressive role of ZBTB46 in the myeloid cell development.

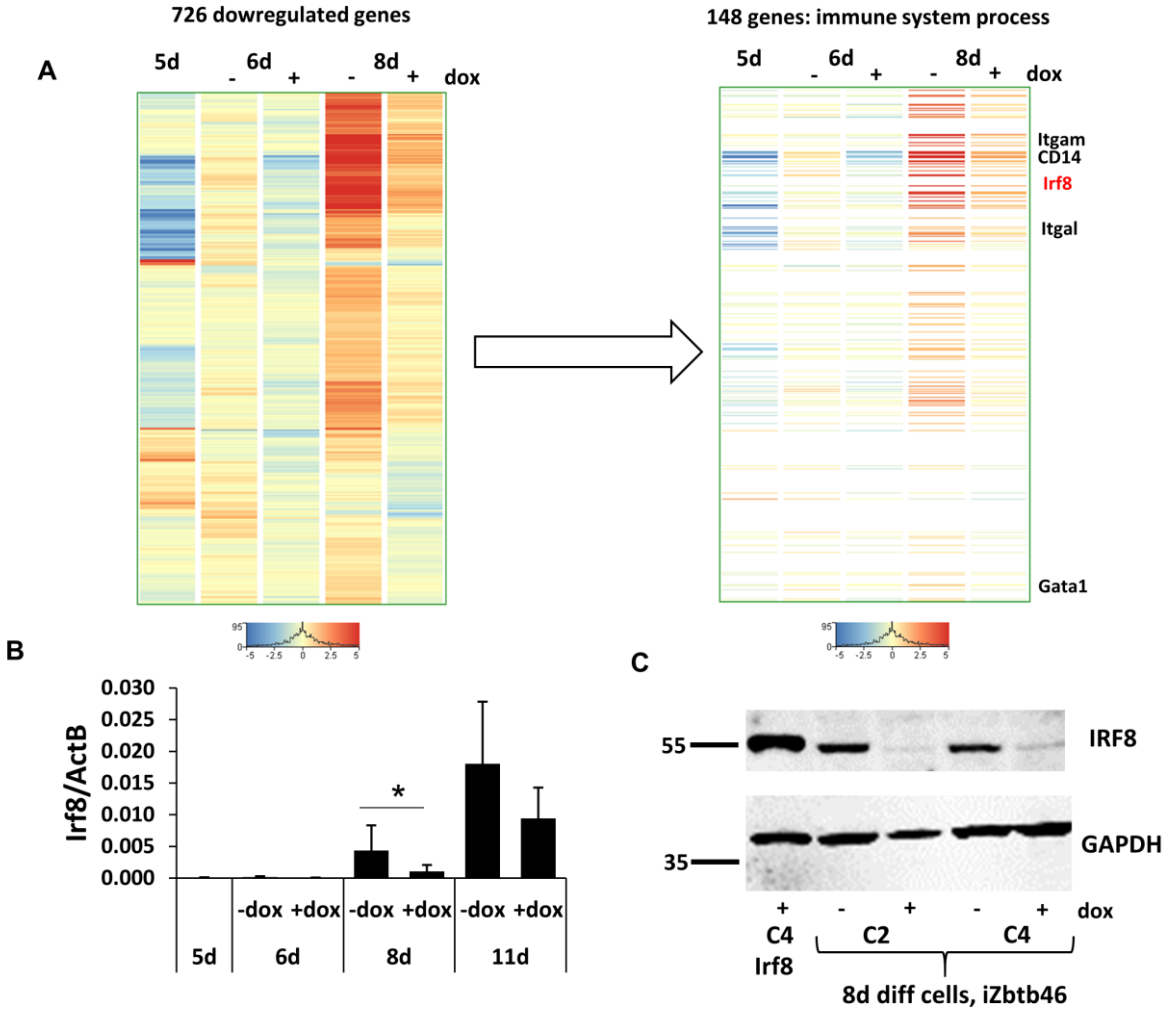

Figure 17. ZBTB46 dependent gene downregulation. A, Hierarchical clustering of 726 transcripts with reduced expression at day 6 and/or 8 upon Zbtb46 induction. Within this gene list the 'immune system process' category was found as a strongly overrepresented gene ontology category; this gene subset contains known regulators of myeloid development. B, Quantitative RT-PCR assay to measure the mRNA level of Irf8. For RT-PCR five day differentiated Flk1+ cells were MACS sorted (5d) and cultured for 1 or 3 additional days (6d or 8d) in the presence of absence of doxycycline. In addition, non-sorted cells were differentiated for 11 days (11d) and the Zbtb46 induction was started at day 5. C, IRF8 protein detection with Western-blot testing 8-day differentiated, Zbtb46-inducible cell clones. The identity of specific bands was confirmed by comigration with a band seen in the extract of Irf8-induced ESCs (Irf8 C4 +dox).

Following these, we assessed those transcript sets with elevated expression level upon ZBTB46 with the help of the same method we used to determine the downregulated genes. We uncovered 361 genes which were positively regulated by ZBTB46, among these entities, our RT-PCR

analyses confirmed two genes (*Cyp26b1* and *Ramp3*) which were upregulated in the ZBTB46 induced cells (**Figure 18**).

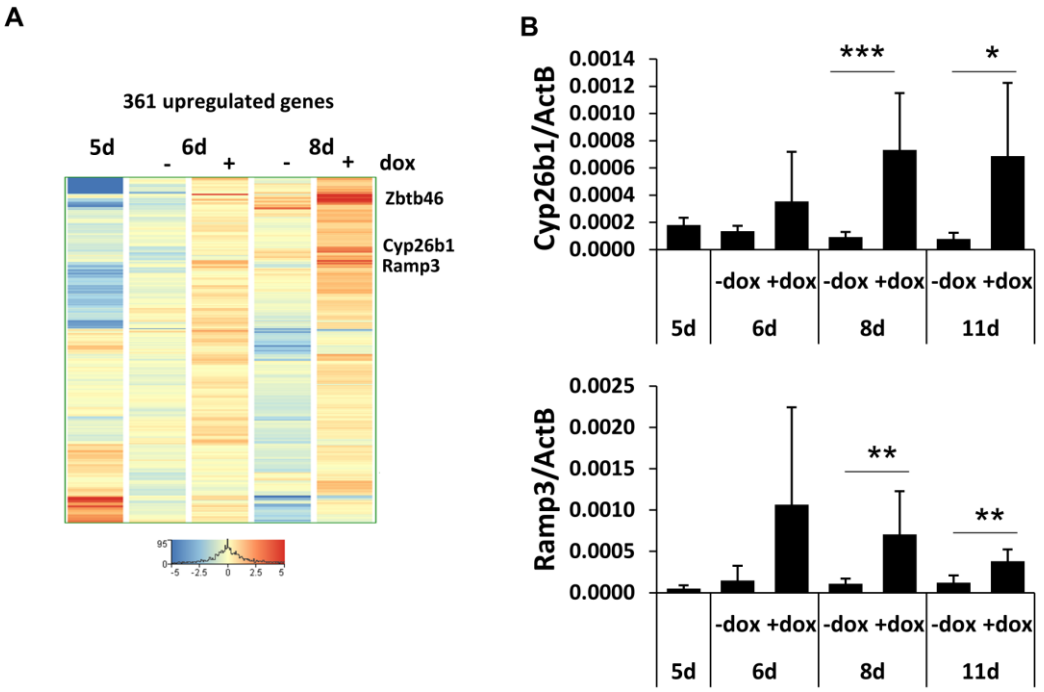

Figure 18. ZBTB46 dependent gene upregulation. A, Hierarchical clustering of 361 transcripts with elevated expression at day 6 and/or 8 upon Zbtb46 induction. B, Transcript levels of Cyp26b1 and Ramp3 were validated with Quantitative RT-PCR. For RT-PCR five day differentiated Flk1+ cells were MACS sorted (5d) and cultured for 1 or 3 additional days (6d or 8d) in the presence of absence of doxycycline. In addition, non-sorted cells were differentiated for 11 days (11d) and the Zbtb46 induction was started at day 5.

5.8 Increased erythroid development by ZBTB46 in ESC originated cells

In this study our transcript analysis revealed the inhibitory role of ZBTB46 to the myelopoiesis in ESC based system. ESC derived progenitors can give rise to other blood cell lineages, for examples, these cells have a known potential to erythroid cell formation. Interestingly, our genome-wide analysis highlighted the increased expression of embryonic and adult hemoglobin genes in 6 and 8 day differentiated cells in comparison with the 5-day differentiated Flk1+ purified cells. In addition to this in 8 day differentiated cell we also identified an elevated transcript levels of Hbb1 and Hbb2 upon priming with ZBTB46 indicating the effect of this transcription factor to the development of red blood cells (**Figure 19**A). Our extended RT-PCR based analysis uncovered the upregulated state of the adult beta globin gene (*Hbb-b1*) in 8 and 11-day differentiated, ZBTB46 instructed cells as well as EBs and their progenitor cells upon overexpression of *Zbtb46* (**Figure 19**B). Contrary to this we failed to detect any change in the expression of the fetal hemoglobin (*Hbb-y*) neither in 8 or 11 day differentiated stage nor in

EBs, however *Hbb-y* was elevated in the presence of ZBTB46 after the formation of EBs and 3-day differentiation. Altogether these results indicate the increased expression of the beta globin gene in ZBTB46 instructed ESC derived cells.

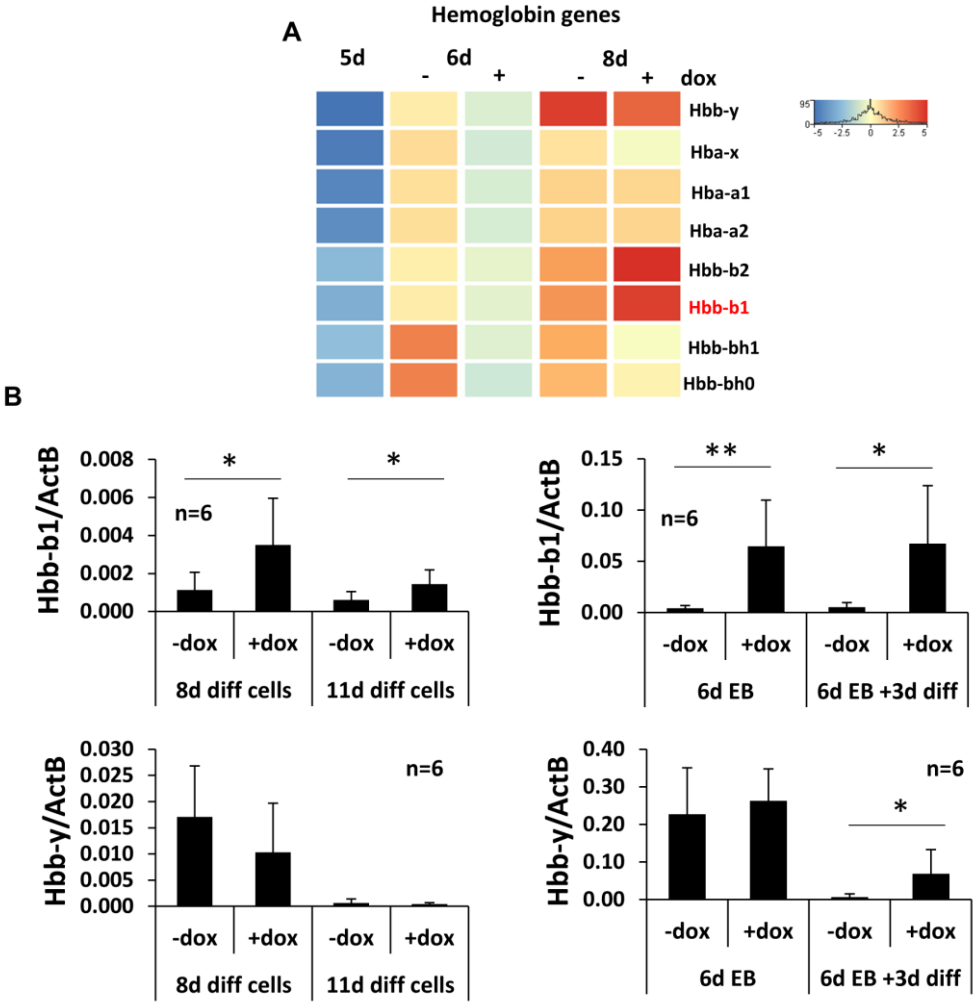

Figure 19. Enhanced hemoglobin expression by Zbtb46. (A) Adult hemoglobin genes (Hbb-b1 and Hbb-b2) show increased mRNA levels upon ZBTB46 induction for 8 days. (B) Validation of Hbb-b1 and Hbb-y mRNA expression with quantitative RT-PCR. Non-purified cells were cultivated for 8 and 11 days and primed with doxycycline as indicated, Zbtb46 was induced from day 5. EBs were cultured for 6 days then further cultured on OP9 feeder cells for 3 days (6d EB + 3d diff). Zbtb46 induction started at day 4.

Following these assessments, we examined the hematopoietic colony forming potential of these cells generated *ex vivo*. The 8 day differentiated cells were harvested then seeded into Methocult M3434 semisolid medium and further cultured for an additional 8 days. At the end the various blood colonies were identified and counted. Strikingly, regardless whether the presence or absence of our transgene, low number of myeloid (GM) and mixed (GEMM) colonies were observed. Contrary to this finding an enhanced number of erythroid colonies were detected upon *Zbtb46* induction. Similarly elevated erythroid cell formation was observed in 11-day

differentiated cells. Development of mixed colonies (GEMM) was also enhanced after *Zbtb46* induction in case of the 11-day differentiated cells. We also observed an impaired number of myeloid colonies (GM) at day 11 and 14 upon introduction of *Zbtb46* (**Figure 20**). Altogether these results suggest that overexpression of *Zbtb46* drives the blood cell development from myeloid to erythroid lineage commitment in our ESC derived progenitor cells.

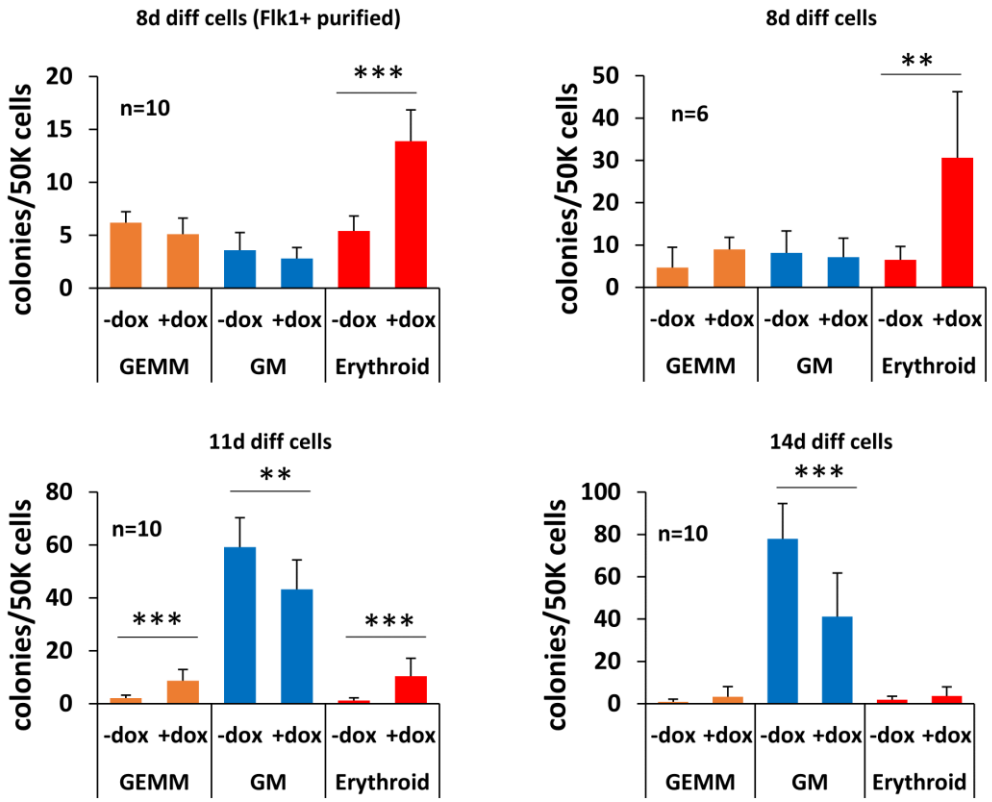

Figure 20. Enhanced erythroid colony formation by ZBTB46. Elevated number of erythroid colonies in 8 and 11-day cultivated ZBTB46 driven cells. At day 8, 11 and 14 the ESC derived cells were cultivated in MethoCult M3434 semisolid medium for additional 8 days to evaluate their ability to form blood cell colonies. The Flk1+ cells were MACS purified at day 5 then further cultivated for 3 days with or without doxycycline.

The major regulators of the erythroid differentiation (KLF1, LMO2, GATA1 and LDB1) however were either repressed or remained unaltered in the presence of ZBTB46 (Figure 17 and data not shown) suggesting the enhanced erythroid development cannot be interpreted solely as result of the general upregulation of the specific regulatory genes and their products. Instead, we observed enhanced CD105 (Endoglin) expression in both the 8-day differentiated cells and EB derived cells upon priming with ZBTB46 (**Figure 21**). This Endoglin positive population was well separated from the CD45+ cells and these two markers were regulated

oppositely by ZBTB46. Being a known marker of erythroid progenitors the enhanced formation of the CD105+ subset can promote the elevated erythroid potential.

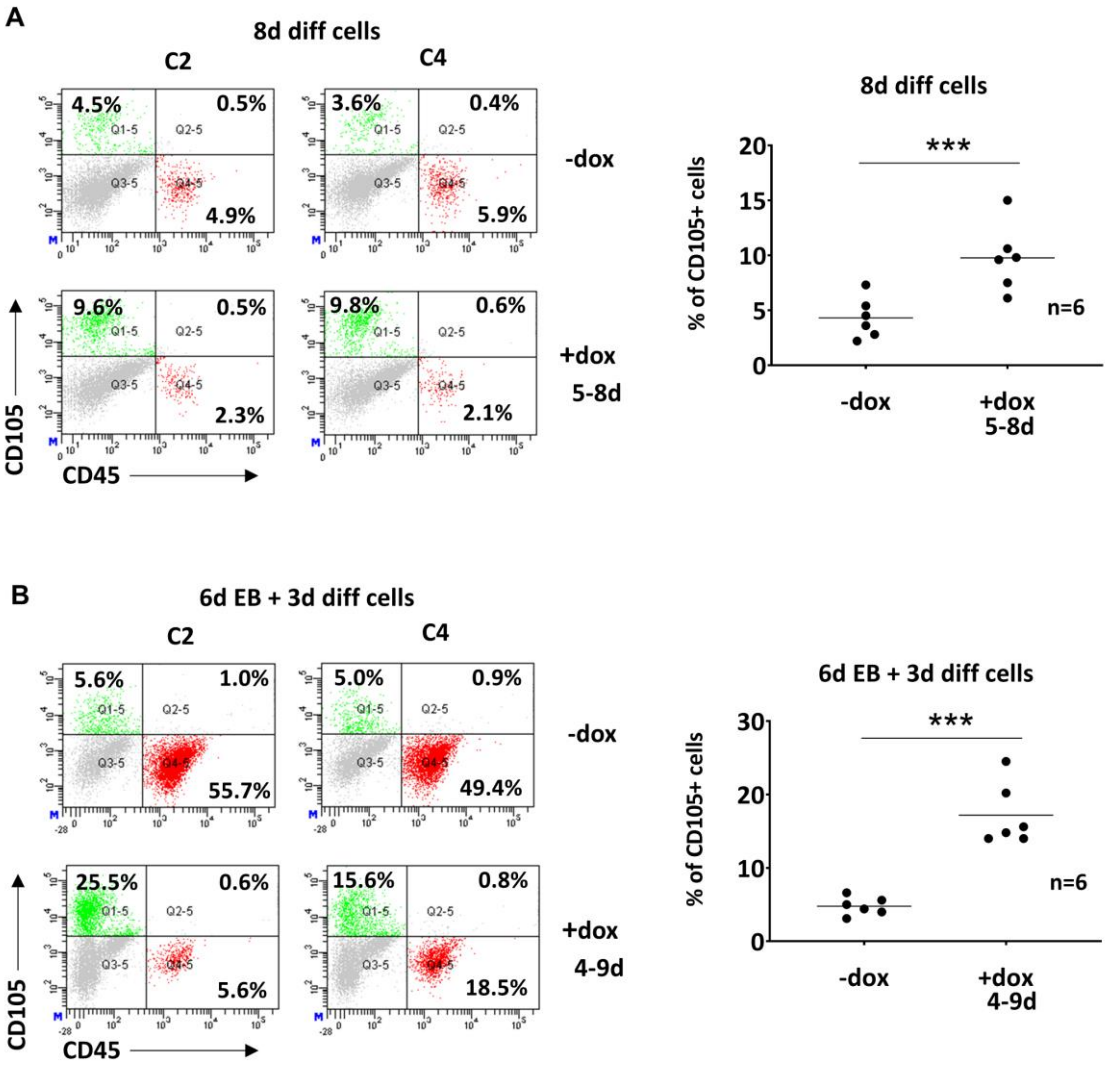

Figure 21. Elevated CD105 expression upon Zbtb46 induction. (A) Day 8; CD105 flow cytometric profile and statistics of the ZBTB46 instructed cells. (B) flow cytometric profile and statistics of 9-day cultivated, Zbtb46 inducible EB derived cells (6d EB + 3d diff). Transgene was induced as indicated by doxycycline (+dox) treatment.

6. Discussion

In our experiments we intended to assess the developmental potential of two DC specific transcription factors, ZBTB46 and RUNX3 during blood cell differentiation of ESCs. Transcription factor assisted cell engineering is frequently applied to modify the cellular identity. This approach can be used to develop special cells for regenerative medicine or immunotherapies. With transcription factors somatic cell can be dedifferentiated into pluripotent cells (iPSC reprogramming) or transdifferentiated into other cell types. In addition, directed differentiation of PSCs can also be facilitated with transcription factors. In case of directed differentiation one can try to recapitulate the embryonic development *ex vivo* towards the desired *in vivo* functional cell types. Importantly, directed differentiation of PSC derived hematopoietic cells have been already enhanced with ectopically expressed transcription factors (Doulatov et al., 2013; Elcheva et al., 2014; Kyba et al., 2002; Sugimura et al., 2017; Wang et al., 2005; Yamamizu et al., 2013). Why do we want to enhance the ES-DC differentiation with transcription factors? There are several protocols for DC differentiation from ESCs or iPSCs (Sachamitr et al., 2014; Senju et al., 2011). These methods employ various cytokines to direct the immune cell formation, especially, GM-CSF are applied to facilitate the myeloid and DC differentiation. Although, it was documented in numerous studies that ES- or iPS-DCs were able to stimulate antitumor immune responses (Senju et al., 2011). However, it was also observed that these ESC or iPSC derived DCs possessed a lower T-cell activation potential than the adult progenitor derived DCs, (Senju et al., 2003; Tseng et al., 2009; Zhang et al., 2015). Consistent with these findings our colleagues also found that ES-DCs showed a lower expression of several maturation specific cell surface markers (MHCII, CD80 and CD86) after LPS exposure compared to BM-DCs (Takacs et al., 2017). These results suggest that some factors are missing from the *ex vivo* generated ESC derived immune cells. Therefore, our colleagues systematically compared the gene expression patterns of numerous DC affiliated transcription factors in ES- and BM-DCs. With RT-PCR analyses Erika Takacs has identified three genes (*Spi-B, Irf4* and *Runx3*) which were underexpressed in ES-DCs versus BM-DCs (Takacs et al., 2017). These results inspired us to use chemically inducible ESC lines to test the effect of these three transcription factors in ES-DCs and their progenitors. To achieve this goal our colleagues engineered Runx3, Irf4 and Spi-B inducible ESC clones using a Cre-mediated site-specific recombination (Bencsik et al., 2016; Takacs et al., 2017). It is worth mentioning that, my colleagues observed a very impaired myeloid blood cell formation in the presence of the *Irf4* transgene, this finding precluded the further analysis of this transcription factor on late-

stage ES-DC development (Takacs et al., 2017). Instead, I analyzed the effects of the transgenic *Spi-B* and *Runx3* on ES-DC activation. Interestingly, the mature ES-DC phenotype remained unaltered in the presence of Irf4 suggesting that this factor has a minimal impact on DC activation. On the other hand, an elevated ratio of MHCII/CD80 double positive cells was found in the RUNX3 instructed ES-DCs in response to LPS priming. Moreover, a unique MHCII expressing cell population was also detected even without LPS activation in the RUNX3 programmed cells. Of note, it was published that *Runx3* is highly expressed in CD11b+ splenic DCs and a cell specific KO model uncovered that the CD11b/Esam expressing DC formation was strongly compromised in *Runx3* null mice (Dicken et al., 2013). Interestingly, in this paper it was also published that the MHCII expression is directly regulated by RUNX3. Our results are consistent with this observation because elevated MHCII positivity was detected in the RUNX3 primed in ES-DCs. However, in our cells the RUNX3-induced phenotypic changes are not confined to the overexpression of MHCII because augmented expression of CD86 was also detected indicating that RUNX3 generally promotes the maturation capacity of ES-DC. In contrast to the cell surface markers, ES-DCs can produce high concentration of proinflammatory cytokines (IL1-β, IL-6 and TNFα) upon LPS exposure regardless of the *Runx3* induction and this cytokine titers were similar in BM-DCs. This finding suggests that the LPS-induced inflammatory cytokine response is probably intact and independent of the *Runx3* expression because the major cytokine production is similar. It seems that some maturation related cell surface marker expressions are selectively modulated by RUNX3 in ES-DCs.

To assess whether RUNX3 can contribute to the functional attributes of our ES-DCs we tested the migratory capacity of the LPS activated ES-DCs using transwell migration assays. Without *Runx3* induction ES-DCs showed an impaired transmigration even in the presence of CCL19 and CCL21. In contrast, the RUNX3 primed cells exhibited a CCL19 and CCL21 dependent enhanced transmigration. Interestingly, the CCL19 and CCL21 chemokines exert their effect on cell migration via the activation of the Ccr7 (C-C motif chemokine receptor 7). One study suggested that in *Runx3* null DCs the Ccr7 expression is lower and these cells has a diminished migratory activity (Fainaru et al., 2005). In contrast, we found an elevated expression of Ccr7 in RUNX3 primed ES-DCs (unpublished observation) suggesting that this chemokine receptor regulation is context and DC subtype specific. We also examined the T cell activation potential of our RUNX3 instructed ES-DCs using MRL reactions. The allogenic stimulation (our antigen presenting cells were co-cultured with T cells which carry a different genetic background) showed an elevated T cell proliferation rate as a response to RUNX3 induced ES-DCs. These

results showed that forced induction of *Runx3* enhanced the T cell activation potential of the ES-DCs. In conclusion, our *ex vivo* analyses revealed that ES-DCs had an inferior migratory and maturation capacity. However, enforced expression of the *Runx3* transgene was sufficient to impart ES-DCs with superior maturation, chemotactic and T cell activation potential. These results suggest that a single transcription factor is enough to improve the ES-DCs maturation and immunogenicity. In the future we intend to test this transcription factor in human PSC derived DC progenitors. It is important to mention that adult progenitor derived DCs (BM-DCs) still contained higher percent of MHCII/CD80 and CD86 positive cells and BM-DCs showed a superior migratory capacity than the RUNX3-primed ES-DCs. These results indicated that RUNX3-induced ES-DCs remained less immunogenic compared to the adult DCs. These results prompt us to test additional transcriptional factors and cytokines along with the RUNX3, to further enhance the immunogenicity of the RUNX3-primed pluripotent stem cell-derived DCs.

In the second part of this study, we investigated the effects of the ZBTB46 protein on ESC differentiation. Similar to RUNX3, the classical DC marker *Zbtb46* was barely expressed in ES-DCs and their progenitors (Takacs et al., 2017) this result inspired us to investigate the effect of this transgene. To uncover the role of this factor in ES-DC development we used the same gain of function approach as we utilized for the RUNX3 analysis. We tested the role of the ZBTB46 in ES-DC differentiation using an OP9 coculture based *ex vivo* differentiation system (Senju et al., 2003) as well as with an EB based differentiation method. Regardless of the applied protocols, ectopic expression of *Zbtb46* invoked a strong repressive effect during the early hematopoietic development. The ESC derived ZBTB46-primed cells inefficiently formed CD45+/CD11b+ myeloid progenitors suggesting that the myeloid blood cell development was repressed by this transcription factor. It is worth mentioning that some inhibitory functions of the ZBTB46 have already been observed. For example, *Zbtb46* null DCs were more activated suggesting that this protein can interfere with the DC maturation (Meredith et al., 2012b). More related to our study, that enforced expression of *Zbtb46* in BM derived hematopoietic progenitors negatively regulated the myeloid granulocyte development and skewed the cell differentiation towards cDC like cells (Satpathy et al., 2012a). Moreover, huge number of monocyte development related genes were upregulated in *Zbtb46* null DCs suggesting that this transcription factor negatively modulates the myeloid/monocytic gene regulatory networks (Meredith et al., 2012b). We also found a reduced myeloid blood cell formation in ESC derived progenitors, however, we did not observe any DC like markers after

the overexpression of *Zbtb46*. These results suggest that ZBTB46 can provoke a general inhibitory effect on the myeloid blood cell development but the direction of the alternative cell development is context dependent. It is well established that multiple members of the ZBTB transcription factor family can elicit suppressive effects during blood cell differentiation (Maeda, 2016). The BTB domains of the ZBTB proteins can directly interact with corepressor complexes and mediates chromatin compaction and gene silencing (Dhordain et al., 1997; Hong et al., 1997; Huynh and Bardwell, 1998; Huynh et al., 2000). It has been already suggested that ZBTB46 can elicit a repressive effect via interaction with corepressor complexes (Meredith et al., 2012b), however, further investigations are needed to prove this possibility. In addition, we proposed that multiple genes and pathways can be repressed by ZBTB46 during the ESC differentiation. Therefore, we examined the genomic impact of this gene regulatory protein with mRNA sequencing. In agreement with the repressive function, we found that more than 700 genes were negatively regulated upon the forced expression of this transcription factor in the 6- or 8-day differentiated cells. Importantly among these transcripts, several myeloid specific mRNAs were detected, including Irf8, CD14, and Itgam (CD11b). It is possible that some of these myeloid specific genes are directly repressed by ZBTB46. Of note, it was already published that numerous myeloid/monocyte specific gene regulatory regions can be occupied with ZBTB46 (Meredith et al., 2012b).

Despite of this strong repressive effects, our genome-wide transcriptional analysis highlighted the elevated expression of embryonic and adult hemoglobin genes in 6 and 8 day differentiated cells compared to the Flk1+ cells differentiated for 5 days. Moreover, elevated level of Hbb1 and Hbb2 was observed upon priming with ZBTB46. We performed an extended analysis using RT-PCR which confirmed the upregulated of the adult hemoglobin gene (Hbb-b1) in 8 and 11 day differentiated cells upon *Zbtb46* induction. The same effect was observed in EBs and EB derived cells upon transgene induction. Consistent with this finding, elevated number of erythroid colonies was detected when we tested ZBTB46 primed 8 or 11 day differentiated cells on a colony forming assay. Together these results indicate that overexpression of *Zbtb46* enhances the erythroid blood cell lineage formation in our ESC based system. Strikingly, the key erythroid regulators (KLF1, LMO2, GATA1 and LDB1) were either unaltered or repressed by ZBTB46. We concluded that the enhanced erythroid development cannot be explained as a result of the upregulation of the erythropoiesis specific transcription factors by ZBTB46. Interestingly, our RNA sequencing analysis revealed that the *Cyp26b1* gene was also upregulated upon the *Zbtb46* induction, and this profile was confirmed with quantitative PCR.

CYP26B1 is involved in the elimination of retinoic acid, therefore, it is possible that this important morphogen is less active in the ZBTB46-primed cells. My colleagues have been previously reported that, retinoic acid interfered with the ESC derived hematopoietic colony formation, including erythroid colony generation (Szatmari et al., 2010). An additional report suggests that this molecule is selectively inhibitory to the erythroid development (Labbaye et al., 1994). Therefore, it is possible that the elevated *Cyp26b1* expression can contribute for the enhanced erythroid development via lowering retinoic acid level. Obviously other mechanisms can also contribute for the ZBTB46 dependent erythroid blood colony formation. For example, we observed an elevated CD105 (Endoglin) expression during the early stage of differentiation in the presence of ZBTB46. The CD105+ cell population was well separated from the CD45+ population and these two markers are oppositely regulated by ZBTB46. It was described that erythroid progenitors express CD105 (Pronk et al., 2007). Although the function of CD105 on erythropoiesis is controversial, but it was also described that the ESC-derived erythroid development is positively modulated by the forced expression of CD105 (Baik et al., 2012). Therefore, the ZBTB46 dependent expanded CD105+ cell population might be connected with the enhanced erythropoiesis. Obviously, further studies will be necessary to characterize the ZBTB46 mediated erythroid development in ESC derived differentiated cells.

As a summary, our analysis illustrate that a single transcription factor (ZBTB46) can overwrite the blood cell developmental program. The overexpression of this transcription factor in mouse ESC-derived cells was sufficient to inhibit the myeloid gene regulatory networks and activate the erythroid pathway. The robust impact of ZBTB46 in ESC derived progenitors suggests that it would be interesting to test other members of the ZBTB proteins alone or in combination to facilitate directed differentiation of ESCs in the future. Moreover, our genome-wide gene expression profiling provides a catalogue for exploration of the ZBTB46 regulatory network in ESC derived progenitors.

7. Summary

The *ex vivo* generated monocyte-derived DCs have been often applied for antitumor immunotherapies. Some of these DC-based vaccines have shown promising clinical results. However, there is a limitation in the number of the harvested monocytes, and the DC-differentiation capacity of these immune cells varies. In contrast to monocytes, the pluripotent ESCs could represent an unlimited source for immunotherapies because of their immortality and broad differentiation capacity. However, it is very challenging to drive the ESC differentiation into DCs because the end products are often immature cells with limited immunogenicity. For ES-DC maturation, further steps are needed which are missing from the existing ESC differentiation protocols. We hypothesized that ectopically-expressed DC specific transcription factors can enhance the maturation of these *ex vivo* differentiated immune cells. In our study we examined the effect of the RUNX3 and the ZBTB46 transcription factors during the mouse ES-DC differentiation and concluded the following results:

1. Mouse ESC derived DCs has a limited maturation capacity, but it can be enhanced by the forced expression of *Runx3*. We obtained more MHCII/CD80 double positive cells as well as more CD86+ cells as response to LPS treatment of the RUNX3-primed cells. In contrast, we found that overexpression of *Spi-B* did not alter the ES-DC maturation.

2. In contrast to the enhanced expression of DC maturation related cell surface markers, RUNX3 cannot influence the LPS induced cytokine production of ES-DCs. However, our results showed an elevated migratory potential of the ES-DCs upon introduction of *Runx3*, but this was still below the benchmark level set by BM-DCs. Importantly, we also found that RUNX3 enhanced the T cell activation capacity of our ES-DCs.

3. We also tested the effect of ZBTB46 during the ES-DC differentiation and failed to generate any fully differentiated DCs in the presence of this transcription factor. Instead of we observed that the enforced expression of *Zbtb46* was associated with a profound inhibition of the myeloid blood cell formation during the ESC differentiation.

4. The mesodermal development was also negatively regulated by ZBTB46, moreover, we revealed that ZBTB46 also negatively influence the cell proliferation probably due to the altered cell cycle profile.

5. Consistent with the suppressed myeloid cell differentiation, our global transcript analysis revealed that several myeloid cell specific genes (for example, *Irf8*, *Itgal* and *Itgam*)

exhibited an impaired expression in the ZBTB46-programmed cells. In addition, we identified numerous mRNA transcripts (for example Cyp26b1 and Ramp3) which were upregulated in the ZBTB46-primed differentiated cells.

6. Finally, we observed that overexpression of Zbtb46 steers the blood cell development from myeloid to the erythroid lineage commitment in ESC derived progenitors. Moreover, we also found that the adult version of the beta globin gene showed an elevated expression in the ZBTB46-instructed ESC-derived progenitors.

In summary, ESC derived DCs had a compromised maturation ability and immunogenicity. However, forced expression of *Runx3* acts as an instructive tool for generation of mature ES-DCs with enhanced immunogenicity. Our findings also illustrate that a single transcription factor (ZBTB46) can overwrite the blood cell development program in ESC derived progenitor: ZBTB46 is sufficient to suppress the myeloid gene regulatory networks and activate the erythroid development.

8. Összefoglalás

Monocita eredetű dendritikus sejteket (DC-ket) gyakran használnak daganatellenes immunoterápia céljából. Fontos kiemelni, hogy a monocita forrásból létrehozott DC alapú vakcinák több esetben ígéretes klinikai eredményekhez vezettek. Ugyanakkor limitált az adott betegből begyűjthető monociták száma, s ezekből nem mindig jön létre terápiás mennyiségű DC. Ellentétben a monocita sejtekkel, a pluripotens embrionális őssejtek (ES sejtek) szinte korlátlan sejtforrást biztosíthatnak mivel az ES sejtek immortálisak és széles fejlődési képességgel rendelkeznek. Ugyanakkor komoly kihívást jelent az ES sejtek irányított átalakítása DC-ké: sok esetben az így létrehozott immunsejtek éretlenek és csak korlátozott immunogenitásuk van. Úgy tűnik további faktorok szükségesek az ES sejt eredetű DC-k (ES-DC-k) maturációjának eléréshez. Hipotézisünk szerin DC specifikus transzkripciós faktorok bekapcsolásával fokozható lehet az ES eredetű immunsejtek maturációja és immunogenitása. Mindezek miatt kísérleteinkben megvizsgáltuk a RUNX3 és a ZBTB46 DC specifikus transzkripciós faktorok hatását az ES-DC-k fejlődése során, és az alábbi következtetéseket vontuk le.

1. Az egér ES sejt eredetű DC-k is limitált maturációs képességgel rendelkeznek, viszont az érett sejtek aránya a RUNX3 transzkripciós faktor bekapcsolásával jelentősen fokozható. A fokozott maturációt támasztja alá, hogy sokkal több MHCII/CD80 dupla pozitív, illetve CD86+ sejt volt megfigyelhető a RUNX3-al átprogramozott ES-DC-ben LPS kezelést követően. Ezzel ellentétben az Spi-B faktor túltermelése nem befolyásolta a DC maturációt, az IRF4 transzkripciós faktor viszont általános gátló hatást gyakorolt az ES eredetű myeloid immunsejtek fejlődésére.

2. A sejtfelszíni markerek vizsgálata alapján tehát azt találtuk, hogy a RUNX3 fokozza az ES eredetű DC-k maturációs képességét, ugyanakkor ezen sejtek LPS indukálta citokin termelése változatlan maradt. További funkcionális vizsgálataink rávilágítottak arra, hogy a RUNX3 pozitívan befolyásolja az ES eredetű DC-k migrációs képességét, viszont ez a képesség még így is elmarad a csontvelői eredetű DC-k hasonló tulajdonságától. Ezen felül igazoltuk, hogy a *Runx3* túltermelése fokozza az ES-DC-k T-sejt aktiváló képességét.

3. A RUNX3 mellett szintén teszteltük a ZBTB46 hatását az ES-DC *ex vivo* differenciáció során. Ezen transzkripciós faktor túltermelése esetén egyáltalán nem jöttek létre DC-szerű sejtek, mivel a myeloid sejtek korai fejlődése szinte teljesen blokkolódott.

4. Megállapítottuk, hogy a ZBTB46 faktor túltermelése nem csak a myeloid vérképző sejtek kialakulását gátolja, hanem az ezt megelőző mezodermális sejtfejlődésre is represszív hatással van. Továbbá ez a fehérje negatívan befolyásolja a sejtproliferációt is, feltételezhetően a sejtciklus szabályozásán keresztül.

5. Összhangban a megfigyelt myeloid sejtfejlődés gátlásával, az általunk végrehajtott globális génexpressziós vizsgálatok feltárták, hogy csökkent expresszió mutat számos myeloid sejt specifikus gén (pl. *Irf8, Itgal* és *Itgam*) is a ZBTB46-al átprogramozott sejtekben. Továbbá olyan géneket is azonosítottunk (pl. *Cyp26b1* és *Ramp3*) melyek expressziója fokozódott ZBTB46 jelenlétében.

6. Végezetül vizsgálataink feltárták, hogy a ZBTB46 transzkripciós faktor túltermelése a myeloid program helyett az erythroid sejtek kialakulását segíti elő az ES sejtek differenciálódása során. Ezzel párhuzamosan az is kimutatható volt, hogy ezek az erythroid sejtek leginkább a felnőtt vörös vérsejtekre jellemző hemoglobin formákat expresszálnak.

Összefoglalásként fontos kiemelni, hogy a pluripotens ES sejtekből létrehozott DC-k immunogenitása korlátozott. Viszont *Runx3* transzgén expressziója esetén olyan ES-DC-k fejlődnek ki, melyek érettebb sejteknek tekinthetők és erőteljesebb immunválaszt tudnak létrehozni. Eredményeink azt is feltárták, hogy a ZBTB46 teljesen képes átírni a korai hematopoiezis folyamatát az ES eredetű progenitorokban, mivel e protein hatására a myeloid sejtek fejlődése represszálódik, ezzel szemben az erythroid sejtek képződése fokozódik.

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11. List of Publications

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Registry number: DEENK/120/2022.PL
Subject: PhD Publication List

Candidate: Pál Botó
Doctoral School: Doctoral School of Molecular Cellular and Immune Biology

List of publications related to the dissertation

1. **Botó, P.**, Gerzsenyi, T. B., Lengyel, A., Szúnyog, B., Szatmári, I.: Zbtb46-dependent altered developmental program in embryonic stem cell-derived blood cell progenitors.
   Stem Cells. 39 (10), 1322-1334, 2021.
   DOI: http://dx.doi.org/10.1002/stem.3424
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2. Takács, E.*, **Botó, P.***, Simó, E., Csuth, T. I., Tóth, B. M., Raveh-Amit, H., Pap, A., Kovács, E. G., Kobolák, J., Benkő, S., Dinnyés, A., Szatmári, I.: Immunogenic Dendritic Cell Generation from Pluripotent Stem Cells by Ectopic Expression of Runx3.
   J. Immunol. 198 (1), 239-248, 2017.
   DOI: http://dx.doi.org/10.4049/jimmunol.1600034
   * These authors contributed equally this work.
   IF: 4.539

List of other publications

3. Douida, A., Batista, F., **Botó, P.**, Regdon, Z., Robaszkiewicz, A., Tar, K.: Cells Lacking PA200 Adapt to Mitochondrial Dysfunction by Enhancing Glycolysis via Distinct Opa1 Processing.
   Int. J. Mol. Sci. 22 (4), 1-22, 2021.
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4. Douida, A., Batista, F., Robaszkiewicz, A., **Botó, P.**, Aladdin, A., Szenykiv, M., Czinege, R., Virág, L., Tar, K.: The proteasome activator PA200 regulates expression of genes involved in cell survival upon selective mitochondrial inhibition in neuroblastoma cells.
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5. Dániel, B., Czimmerer, Z., Halász, L., **Botó, P.**, Kolostyák, Z., Póliska, S., Berger, W. K., Tzerpos, P., Nagy, G., Horváth, A., Hajas, G., Silye-Cseh, T., Nagy, A., Sauer, S., Francois-Deleuze, J., Szatmári, I., Bácsi, A., Nagy, L.: The transcription factor EGR2 is the molecular linchpin connecting STAT6 activation to the late, stable epigenomic program of alternative macrophage polarization.
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9. Bencsik, R., **Botó, P.**, Szabó, R. N., Tóth, B. M., Simó, E., Bálint, B. L., Szatmári, I.: Improved transgene expression in doxycycline-inducible embryonic stem cells by repeated chemical selection or cell sorting.
Stem Cell Res. 17 (2), 228-234, 2016.
DOI: http://dx.doi.org/10.1016/j.scr.2016.08.014
IF: 3.494

Total IF of journals (all publications): 52,155
Total IF of journals (publications related to the dissertation): 10,816

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The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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